Docket No.: P0786.70000US05
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	John B. Sullivan et al.
Serial No.:	08/405,454
Confirmation No.:	6004
Filed:	March 15, 1995
For:	ANTIVENOM COMPOSITION CONTAINING FAB FRAGMENTS
Examiner:	R. B. Schwadron
Art Unit:	1644

DECLARATION OF RICHARD C. DART, M.D., PH.D.

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Richard C. Dart, hereby declare as follows:

1. I am currently the Director of the Rocky Mountain Poison and Drug Center, a position I have held since 1992. I am also a Professor of Surgery (Emergency Medicine), Pharmacy, and Medicine at the University of Colorado Health Sciences Center.

2. From 1986 to 1991, I was a doctoral student at the University of Arizona College of Pharmacy, receiving a Ph.D. in Pharmacology/Toxicology in 1991.

3. From 1985 to 1987, I was a Clinical Toxicology Fellow at the University of Arizona Health Sciences Center. My fellowship director was Dr. John B. Sullivan, and I worked there with Dr. Findlay E. Russell, who are the two inventors of this Application

4. From 1983 to 1985, I was a Resident in Emergency Medicine at the University of Arizona Health Sciences Center, Tucson, AZ.

5. From 1982 to 1983, I was a Resident in Internal Medicine at the Albany Medical Center, Albany, NY.

6. From 1981 to 1982, I was an Intern in Internal Medicine at the Albany Medical Center, Albany, NY.

7. From 1977-1981, I was a medical student at Wayne State University School of Medicine, Detroit, MI, receiving an MD in 1981.

8. I received a BS. in Biology from Albion College, Albion, MI in 1977.

9. As shown in my attached C.V. [Ex. 1], I have published over 175 articles, chapters, editorials, and letters. I am the editor of *The Five Minute Toxicology Consult* (First Ed.) and *Medical Toxicology* (Third Ed.), which has been called the industry's reference and text book.

10. I was the 2004 recipient of the American College of Medical Toxicology Matthew J. Ellenhorn Award for Excellence in Medical Toxicology and am currently President-Elect of the American Association of Poison Control Centers.

11. Much of my clinical, consulting, and research experience relates to the treatment of snake bites with antivenoms. I have served as Principal Investigator for several clinical trials on different antidotes, I have consulted for several different biopharma companies (including the assignee, Protherics, Inc.) regarding antivenom use and development, and I continue to do so. I was the Principal Investigator for the clinical trial of CroFab that ultimately resulted in it being approved by the FDA. For this research, I was awarded the Food And Drug Administration's Special Citation in 2001.

12. The antivenom field is relatively small, and I consider myself to be an expert on the topic. I also consider myself to have been one of ordinary skill in the field as of October 9, 1984. At that time I was over 3 years into my career as an M.D., including one year as a Resident in Emergency Medicine at the University of Arizona Health Sciences Center, whose Emergency Room treats as many snake bite victims as any other Emergency Room in the United States. I was also preparing to start my Fellowship in Clinical Toxicology.

13. I have been retained as a testifying expert by Wolf Greenfield & Sacks, the law firm representing the assignee Protherics, Inc. in this matter. The Rocky Mountain Poison and Drug Center is being paid at the rate of $500 per hour for my time spent reviewing materials, consulting, and preparing this declaration. I have no financial interest in whether a patent issues from this application.

14. I have read the Decision on Appeal dated June 15, 2009. [Ex. 2.] I disagree with its conclusion that one of ordinary skill in the art would have been motivated to prepare an antivenom pharmaceutical composition for treating a snakebite victim, comprising Fab fragments which bind specifically to a venom of a snake of the Crotalus genus and a pharmaceutically acceptable carrier, with a reasonable expectation that the composition would neutralize the lethality of the venom of a snake of the Crotalus genus. [Ex. 2 at p. 16, second full paragraph; p. 16, last paragraph, through page 17, first full paragraph.]

15. I first note that the Decision bases its conclusion on the assertion that the Fab teachings of the Coulter et al. article [Ex. 3]would have led one of ordinary skill in the art to prepare Fab fragments of the affinity-purified antibodies of the Sullivan et al. article [Ex. 4] because the Fab fragments would be expected to have improved sensitivity in assays. [Ex. 2 at p. 16, first full paragraph.] I simply do not understand how this is relevant to the claimed invention, which I understand is:

> 40. An antivenom pharmaceutical composition for treating a snakebite victim, comprising Fab fragments which bind specifically to a venom of a snake of the Crotalus genus and which are essentially free from contaminating Fc as

> determined by immunoelectrophoresis using anti-Fc antibodies, and a pharmaceutically acceptable carrier, wherein said antivenom pharmaceutical composition neutralizes the lethality of the venom of a snake of the Crotalus genus

16. The Decision seems to ignore that this claim concerns an antivenom, not an antibody for an immunoassay. The Decision's posited immunoassay reagent would not be an "antivenom." It would not be a "pharmaceutical composition." It certainly would not be used by any clinician "for treating a snakebite victim." And no clinician would reasonably expect such an immunoassay reagent to "neutralize the lethality of the venom of a snake of the Crotalus genus."

17. It has been explained to me that the Decision refused to consider these elements of the claimed invention based on some legal analysis that is beyond my training and expertise. Regardless of any legal analysis, as one of at least ordinary skill in the art, I read claim 40 as requiring an *in vivo* purpose, use, property, and effect. Nothing in the combined teachings of the Sullivan et al. and Coulter et al. articles would have provided a reasonable expectation of "neutralizing the lethality of the venom of a snake of the Crotalus genus" with an "antivenom pharmaceutical composition" "for treating a snakebite victim" comprising Fab fragments that bound to the venom of a snake of the Crotalus genus.

18. The Decision misapprehends the Coulter et al. article. Coulter et al. showed that Fabs raised to a single toxin "isolated from the venom of the Australian brown snake, *Psuedonaja textilis*" [Ex. 3 at p. 199, last sentence] neutralized the lethality **of that single toxin**. [Ex. 3 at p. 201 third full paragraph.] Coulter et al. raised Fab fragments to textilotoxin (not whole venom) and tested those Fab fragments "for their ability to neutralize the lethal effects of textilotoxin [not whole venom] in mice." [Ex. 3 at p. 201 third full paragraph.]

19. The Decision repeatedly mischaracterizes the teachings of the Coulter et al. article, confusing the distinctions between an individual toxin of a snake venom and the entire snake venom. For example, the Decision states:

> Coulter provides the evidence necessary to establish that Fab fragments are effective in neutralizing the toxicity of snake **venom**. [Ex. 2 at p. 20, first paragraph (emphasis added).]
>
> Coulter teaches that Fab fragments are effective in neutralizing the toxicity of snake **venom**. [Ex. 2 at sentence bridging pp. 20-21 (emphasis added).]
>
> Coulter took that step and taught that Fab fragments are effective in neutralizing the toxicity of snake **venom**. [Ex. 2 at p. 21, last paragraph (emphasis added).]
>
> Coulter teaches that Fab fragments are effective in neutralizing the toxicity of snake **venom**. [Ex. 2 at p. 23, second full paragraph (emphasis added).]

20. None of these statements is true. The Coulter et al. article taught that Fab fragments were effective in neutralizing the lethality of a single venom toxin, **not** an entire snake venom. Appellants were the first to teach or suggest neutralizing the lethality of an entire snake venom with an antivenom comprising Fab fragments.

21. The combined teachings of the Coulter et al. article and the Sullivan et al. article would not have provided one of ordinary skill in the art with a reasonable expectation that Fabs could neutralize the lethality of:

> 1) *Psuedonaja textilis* venom as a whole by administering Fabs raised to just textilotoxin;
>
> 2) *Psuedonaja textilis* venom as a whole by administering Fabs raised to the entire venom; or
>
> 3) *Crotalus* venom as a whole by administering Fabs raised to *Crotalus* venom.

22. First, snake venoms are very complex mixtures of small and large molecules, including numerous toxins. They are so complex that most have not had all their components fully characterized, despite decades of research. Similarly, the properties of most venom components were not known in 1984, despite decades of research. However, many of the most toxic components of snake venoms have been identified and their properties generally classified. Thus, these toxins are sometimes referred to as, for example, neurotoxins, cardiotoxins, hemorrhagics, and fibrinolytics. These properties are not necessarily exclusive, and a particular toxin may have more

than one of these properties. Moreover, the individual toxins can interact synergistically with other toxins in a venom.

23. Nonetheless, most medically important venoms have been characterized in terms of the main toxic effect of their most clinically significant individual toxins, which can sometimes comprise a small percentage of a venom's total individual toxins. An antivenom must neutralize all of these clinically important toxins of a venom to neutralize the lethality of that venom. [Ex 5 at p. 83 ("An antivenom must be capable of neutralizing the injurious components of the venom."); 85 ("Thus, there may be a limited number of clinically important components that require neutralization."). Neutralizing the lethality of one toxin is not effective since other clinically important toxins could still cause lethality. [Ex. 6 at p. 319 col. 2 ("venoms are complex mixtures of proteins and other toxic factors could cause death.") ("Both the hemorrhagic and fibrinolytic activities need to be neutralized with antivenom.").]

24. *Psuedonaja textilis* was known in 1984 to have several clinically important toxins. Neutralization of the lethality of only one of those toxins, as shown in the Coulter et al. article, would not have been expected to result in neutralizing the lethality of the entire venom because the other lethal toxins would remain unneutralized. Indeed, the existing *Psuedonaja textilis* antivenom suffers from this very problem. It neutralizes the activity of textilotoxin, but it does not sufficiently neutralize the prothrombin activator, leading to coagulopathy and potentially fatal cerebral hemorrhage. [Ex. 7 at p. 80, first full paragraph. ("While CSL Ltd. antivenoms have saved many lives, persistent difficulties are being experienced with its inability to efficiently reverse the effects of the prothrombin activator. Unrelenting coagulopathy due to the slow reversal of prothrombin activator presents the added risk of cerebral hemorrhage to the victim.").] One of ordinary skill in the art would not have expected Coulter et al.'s Fab fragments to textilotoxin to have neutralized the lethality of the entire venom of *Psuedonaja textilis*. Neutralizing one weapon in the venom's arsenal of lethal toxins would not neutralize the activity of its other lethal toxins.

25. Second, one of ordinary skill in the art also would not have expected application of Coulter et al.'s Fab method to the entire venom to yield an antivenom that neutralized the lethality of

Psuedonaja textilis venom. Given the diversity in size, charge, and structure of snake venom toxins, Coulter et al.'s ability to obtain Fab fragments that neutralized the lethality of textilotixin would not have provided a reasonable expectation that one of ordinary skill in the art could have obtained Fab fragments that neutralized the lethality of the other clinically significant *Psuedonaja textilis* venom toxins.

26. An Fab fragment neutralizes the lethality of a venom toxin by binding to the toxin in such a way that it blocks the binding of the toxin to its target. The Fab can itself block the binding of the toxin via stearic hindrance (physically or by polarity), or the Fab can alter the structure of the toxin. In either case, the neutralization requires a specific binding between the Fab and the toxin. The Fab must have a specific 3-D structure and charge to bind the toxin so that it blocks its binding to the target. Otherwise, the Fab can bind to the toxin but have no effect on its activity. [Ex. 5 at p. 86 ("it is crucial to understand that binding of a venom component does not necessarily mean neutralization.").]

27. The only commonality between textilotoxin and other clinically significant *Psuedonaja textilis* venom toxins is that they are contained in *Psuedonaja textilis* venom. Like all snake venoms, *Psuedonaja textilis* venom is a complex mixture of very different molecules. Coulter et al.'s teaching that Fab could neutralize the lethality of textilotoxin would have provided no more guidance on the ability of Fabs to neutralize all the other clinically significant *Psuedonaja textilis* venom toxins than it provided on the ability of Fabs to neutralize any other combination of toxins. Indeed, I am not aware of Coulter et al. ever producing a *Psuedonaja textilis* antivenom comprising Fab fragments despite the great commercial importance of *Psuedonaja textilis* antivenom to their employer, CSL Laboratories, which produced several *Psuedonaja* antivenoms.

28. Third, even if Coulter et al. did produce an antivenom that neutralized the lethality of Psuedonaja textilis venom, one of ordinary skill in the art would not have had a reasonable expectation of success in extrapolating results with an antivenom to *Psuedonaja textilis* venom to an antivenom to Crotalus venom. The snakes are in two different genera—Psuedonaja and Crotalus. Indeed they are in two different families—Elapidae and Crotalidae. There are significant

differences between the venoms of those two families. The venom of elapids, while a complex mixture of chemicals, is relatively simple for snake venom. The venom of Crotalids, however, is extremely complex. Indeed, while Psuedonaja venoms can have 3-4 lethal toxins, Crotalus venoms have at least 6 lethal toxins. One of ordinary skill in the art would not have extrapolated antivenom results involving a single toxin of a relatively simple Psuedonaja venom to predict with any reasonable expectation of success what would happen with an antivenom to a much more complex Crotalus venom.

29. Immune reactions to Wyeth's Antivenin (Crotalidae) Polyvalent (ACP) had long been known to be a problem. The problem was so great that some clinicians refused to give ACP, and others felt stuck between the rock of not treating a snake bite victim with an antivenom and the hard place of treating a snake bite victim with an antivenom that might be worse for the victim than the venom being treated. The immune reactions were mainly attributed to 1) extraneous protein in the antivenom and 2) the presence of the Fc portion of the IgG molecules. The Sullivan et al. article addressed the first cause of those reactions by affinity purifying the IgG molecules that actually bound four target *Crotalus* venoms. [Ex. 4.] Before Applicants' invention, nobody had addressed the second aspect of this long-felt need for a safer antivenom, despite the major concern clinicians had regarding allergic reactions to ACP. I believe this is why the FDA granted CroFab orphan drug status.

30. Fab fragments had long been known to have potential application as an antidote, dating back at least to the use of Fabs to treat digoxin overdose in 1971. [Ex. 8 at p. 385, first paragraph.] And many antivenoms that eliminated the Fc fragment had been made and used. Those antivenoms, however, comprised $F(ab)_2$ fragments, not Fab fragments. $F(ab)_2$ fragments differ from Fab fragments by being split from the Fc portion below the hinge rather than above the hinge. The result is that $F(ab)_2$ fragments comprise two antigen binding sites, still joined at the hinge, while Fab fragments split into two separate binding sites. Despite the relatively widespread use of antivenoms comprising $F(ab)_2$ fragments, particularly in Australia, nobody prepared an antivenom comprising Fab fragments before the Applicants.

31. I believe nobody progressed to the smaller Fab fragments for two reasons. First, the $F(ab)_2$ fragment antivenoms were not as safe as had been anticipated, still resulting in allergic reaction in 30-84% of cases. [Ex. 5 at p. 90, second full paragraph.] Second, the bivalency of $F(ab)_2$ fragments allows them to bind and cross-link two toxins, often resulting in a large $F(ab)_2$-toxin complex being precipitated out of solution; monovalent Fab fragments cannot do that. The less than expected increase in safety of $F(ab)_2$ fragment antivenoms, combined with this potential for lower effectiveness of Fab fragment antivenoms, prevented those skilled in the art from proceeding to Fab fragment antivenoms.

32. Despite those concerns, the Applicants prepared a Fab fragment antivenom and tested its ability to neutralize the lethality of Crotalus venom. Unexpectedly, they found that the Fab fragment antivenom not only neutralized the lethality of Crotalus venom, but it did so both better than ACP and better than antivenom purified according to the Sullivan et al. article. Table 1 shows that the Fab fragment antivenom protected 6 of 9 mice from death, while ACP protected only 3 of 9. [Ex. 9 at p. 19.] Table 2 shows that the Fab fragment antivenom protected 2 of 4 mice from death, while ACP protected only 1 of 4. [Ex. 9 at p. 20.] Table 3 shows that the Fab fragment antivenom protected 4 of 4 mice from death, as did the antivenom prepared according to the Sullivan et al. article, while ACP protected only 1 of 4. [Ex. 9 at p. 20.] Table 4 shows that the Fab fragment antivenom significantly delayed the time of death in mice given a dose that is lethal in 99% of subjects, compared to both the antivenom prepared according to the Sullivan et al. article, and ACP. [Ex. 9 at pp. 20-21.] Table 5 shows that the Fab fragment antivenom protected 5 of 5 mice from death while the antivenom prepared according to the Sullivan et al. article protected 3 of 5, and ACP protected 0 of 5. [Ex. 9 at p. 21.] Finally, Table 6 shows that the Fab fragment antivenom significantly delayed the time of death in mice given a dose that is lethal in 99% of subjects, compared to both the antivenom prepared according to the Sullivan et al. article, and ACP. [Ex. 9 at p. 22.]

33. Even if one of ordinary skill in the art were to read the Coulter et al. article as the Decision did—ignoring the very real and clinically important distinction between neutralizing a toxin of a venom and neutralizing the entire venom—these results are unexpected based on the Coulter et al.

article. The Coulter et al. article reported that Fab had the equivalent neutralization ability as its corresponding IgG **on a weight basis**. [Ex. 3 at p. 202, third paragraph.] IgG has a mass of approximately 150 kDa, while Fab has a mass of approximately 50 kDa. Thus, 3 times as many Fab fragments need to be given to have the same neutralizing ability as IgG according to the Coulter et al. article. Indeed, the Coulter et al. article concludes that "Fab fragments can be obtained from rabbit IgG **with losses of 20-30% of initial IgG antibody activity**." [Ex. 3 at p. 202, last paragraph (emphasis added).] Applicants' results surprisingly do not show such a loss in neutralizing ability for an Fab fragment antivenom. Instead, they show an increase in neutralizing ability for an Fab fragment antivenom.

34. This unexpected increase in effectiveness, combined with the increased safety, greatly interested clinicians in the field. CroFab, the commercial embodiment of the claimed invention, was first scientifically reported in the Consroe et al. article in 1995. [Ex. 10.] That article reported no adverse reactions in mice treated with CroFab. [Ex. 10 at p. 509, col. 1.] In line with the surprising results reported in the Application, it also reported that CroFab was on average 5.2 times more potent than ACP. [Ex. 10 at p. 509, col. 1.]

35. Two years later, we reported the results from the first CroFab clinical trial, demonstrating that CroFab was in fact safe and effective in a clinical setting. [Ex. 11.] CroFab was not approved until October 2000. After our 1997 article, I received many emails from clinicians asking when CroFab would be available, and I continued to receive that question whenever I attended professional meetings. Clinicians were clamoring to get CroFab. The consensus among the inquiring clinicians, which I shared, was that CroFab was so vastly superior to ACP in safety and in efficacy that it would completely supplant ACP in the market.

36. That is in fact what happened. CroFab could not be produced fast enough to meet the initial demand, and Wyeth announced that it was going to discontinue production of ACP within a year of CroFab's launch. [Ex. 12 at p. 32.] I recall Wyeth being vague about why they were discontinuing ACP, but those in the field viewed it as a recognition of what we all felt at the time; CroFab was so vastly superior to ACP that we all wanted to use CroFab if given a choice.

37. The essentially immediate substitution of CroFab for ACP is particularly striking in light of the significant cost premium for CroFab. CroFab was more expensive per vial than ACP, and treatment requires more vials than ACP due to its shorter half-life. Standard dosing for a moderate envenomation with ACP would cost a hospital $3,812.50-$6,862.60, while treatment with CroFab would cost the hospital $10,750-$19,350—almost 3 times as much. [Ex. 13 at pp. 225-226.] Despite that significant cost premium, clinicians pushed their hospitals to stock CroFab and stopped ordering ACP soon after the launch of CroFab.

38. For the above reasons, I believe that the Decision does not reflect how those of ordinary skill in the art would have viewed the claimed invention, nor does it reflect how those of ordinary skill in the art would have viewed the teachings of the prior art. The Sullivan et al. and Coulter et al. articles, and any other articles I know, would not have provided one of ordinary skill in the art with a reasonable expectation that an antivenom pharmaceutical composition for treating a snakebite victim, comprising Fab fragments which bind specifically to a venom of a snake of the Crotalus genus and a pharmaceutically acceptable carrier, would neutralize the lethality of the venom of a snake of the Crotalus genus.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 6/8/10

Richard C. Dart, M.D., Ph.D.

RICHARD CHARLES DART, MD, PhD

PERSONAL DATA

Date of Birth: March 31, 1955

Place of Birth: Detroit, Michigan

Address: Rocky Mountain Poison and Drug Center
777 Bannock St., MC 0180
Denver, CO 80204

Telephone: 303-739-1240 (work)
303-873-0369 (home)

Citizenship: United States of America

PRESENT POSITIONS:

Director, Rocky Mountain Poison & Drug Center
Denver Health Authority
And
Chief Business Development Officer
Denver Health and Hospital Authority
777 Bannock Street, MC 0180
Denver, Colorado 80204

Professor of Surgery (Emergency Medicine)
University of Colorado Health Sciences Center
4200 E. Ninth Avenue
Denver, Colorado 80262

Chief, Medical Toxicology Service
Denver Health Medical Center
777 Bannock Street
Denver, Colorado 80204

EDUCATION

Undergraduate School: Albion College, Albion, Michigan, B.A. Biology; summa cum laude, 1977

Medical School: Wayne State University School of Medicine, Detroit, Michigan, M.D.; 1981

Internship: Albany Medical Center, Albany, New York, Internal Medicine; July 1981 - June 1982

Residency:	Albany Medical Center, Albany, New York, Internal Medicine; July 1982 - June 1983
	University of Arizona Health Sciences Center Tucson, Arizona, Emergency Medicine; July 1983 - June 1985
	Chief Resident, Emergency Medicine; July 1984-June 1985
	Fellowship Emergency Medicine Clinical Toxicology University of Arizona Section of Emergency Medicine; July 1985 - June 1987
Advanced Postgraduate Training:	University of Arizona, College of Pharmacy, Doctoral Dissertation, Pharmacology/Toxicology, "Hepatic injury and lipid peroxidation in hemorrhagic shock and resuscitation." 1986-1991.

ACADEMIC APPOINTMENTS

Professor	Section of Emergency Medicine, Department of Surgery University of Colorado Health Sciences Center 4200 E. Ninth Avenue Denver, Colorado 80262 October 2001 - current.
Associate Professor of Pharmacy, Adjunct	Toxicology Program University of Colorado Health Sciences Center 4200 E. Ninth Avenue Denver, Colorado 80262 August 1, 2003 – current
Faculty Appointment	Clinical Sciences Program University of Colorado Health Sciences Center 4200 E. Ninth Avenue Denver, Colorado 80262 July 2, 2004 - current
Associate Professor, Section of Emergency Medicine	Department of Surgery University of Colorado Health Sciences Center 4200 E. Ninth Avenue Denver, Colorado 80262 October 1992 – 2001
Adjunct Clinical Faculty	School of Pharmacy University of Wyoming 1000 E. University Avenue Laramie, WY 82071 June 2007 - current

Clinical Lecturer	Department of Surgery University of Arizona October 1992 - Current.
Fellowship Director	Clinical Toxicology Fellowship 1989-1991, University of Arizona.
Assistant Professor	Section of Emergency Medicine, Department of Surgery, Tenure Track University of Arizona, June 1991 - October 1992.
Instructor	Section of Emergency Medicine, Department of Surgery, Tenure Track July 1987 - June 1991.

OTHER PROFESSIONAL EXPERIENCE

Staff Physician, Emergency Department, University Hospital, 1992 - 1996.

Staff Physician, Internal Medicine (Medical Toxicology), Denver Health Medical Center, 1992 - current

Medical Consultant, Venom Research Laboratory, Veterans Administration Hospital, Salt Lake City, Utah.

Medical Consultant, Denver Zoological Foundation, Denver, Colorado.

Associate Medical Director and Attending Physician Arizona Poison and Drug Information Center. July 1987 - 1992.

Attending Physician - Section of Emergency Medicine, University of Arizona Health Sciences Center, July 1985 - 1992.

Director, Clinical Toxicology Program, University Medical Center, Tucson, Arizona, 1991 - 1992.

BOARDS

American Association of Poison Control Centers, President-elect, 2008-2010.

American Association of Poison Control Centers, Treasurer, 2004-2006, 2006-2008.

American Board of Emergency Medicine, Medical Toxicology Sub-Board Member, 1993-1998.

American Association of Poison Control Centers, Board of Directors, 1994-2000.

Advisory Board, American Pharmaceutical Association, Responsible Use of Nonprescription Products, 1998 - 2000.

American College of Medical Toxicology, Board of Directors, 1993 - 1996.

Advisory Board, Occupational Medicine Program, University of Arizona, 1991-1992.

Medical Advisory Board, Remote Area Medical Volunteer Corp. Provides expert volunteers from the medical, natural resource, veterinary, economic and agricultural fields to rural populations.

LICENSURE/CERTIFICATIONS

Medical License	Colorado 1992 - Current
	Arizona 1986 - Current
	Idaho 1998 - Current

Recertification - American Board of Emergency Medicine, 1996 and 2006.

Diplomate - American Board of Emergency Medicine, November 23, 1986.

Diplomate - American Board of Medical Toxicology September 30, 1988.

Fellow - American College of Emergency Physicians, 1990.

Fellow – American College of Medical Toxicology, 1996.

HONORS

Summa Cum Laude - Albion College 1977.

Phi Beta Kappa - Albion College 1977.

American College of Emergency Physicians Research Scholar, 1985-1986.

Emergency Medicine Foundation/McNeil Consumer Products Research Scholar, 1986-1987.

Emergency Medicine Foundation/McNeil Consumer Products Research Scholar, 1987-1988.

National Institutes of Health Individual Postdoctoral Fellowship Award, 1988-1990.

Best Scientific Paper, 1994. Dart RC, Seifert SA, Carroll L, et al. Human multicenter trial of an affinity purified antibody fragment for snake venom poisoning. American Association of Poison Control Centers.

Food and Drug Administration (FDA) 2001. Commissioner's Special Citation for contribution to the development of CroFab antivenom to treat patients with North American crotalid snake envenomation.

American College of Medical Toxicology. Matthew J. Ellenhorn Award for Excellence in Medical Toxicology, 2004.

University of Colorado Health Sciences Center Clinical Sciences Program Outstanding Research Mentor Award, 2005.

EDITORIAL BOARDS

Editorial Board, Toxicology Section Editor, Annals of Emergency Medicine, 1992 – 2007. Deputy Editor 2007-current.

Editorial Board, Toxicology Section Editor, Journal of Intensive Care Medicine, 1992 - 1998.

Guest Editor, "Case Reports in Environmental Medicine - Gasoline Toxicity", Agency for Toxic Substances and Disease Registry.

Associate Editor, POISINDEX Information System, 1990 - current.

Co-Editor, Abstract Section, Annals of Emergency Medicine, 1990 - 1992.

Guest Editor, Annals Collector Series on Toxicology. Annals of Emergency Medicine, 1993.

Guest Editor, Toxicology of Metals, Journal of Toxicology - Clinical Toxicology, 1992.

Manuscript Reviewer for:

New England Journal of Medicine
Journal of the American Medical Association
Annals of Emergency Medicine
Archives of Internal Medicine
Public Library of Science (PLOS)
Life Sciences
American Journal of Tropical Medicine
Toxicon
Journal of Toxicology - Clinical Toxicology
Journal of Pharmacology and Experimental Therapeutics

ORGANIZATIONS

American Academy of Clinical Toxicology
American Association of Poison Control Centers
American College of Emergency Physicians
International Society of Toxinology
Society for Academic Emergency Medicine
The Futurist Society
Drug Information Association
Society of Toxicology
College on Problems of Drug Dependence

COMMITTEES - HOSPITALS

Executive Committee, Denver Health and Hospitals, 2008-current

President, Denver Health Medical Staff, 2006-2008.

Chairman, Pharmacy Task Force, Denver Health Medical Center, 1994-current.

Chairman, Pharmacy and Therapeutics Committee, Denver Health Medical Center, 1995 - current.

COMMITTEES - STATE

Drug Box Committee, Arizona Department of Health Services Emergency Medical Services, 1989-1992.

COMMITTEES - NATIONAL

American Association of Poison Control Centers, Finance and Audit Committee, Chair, 2006-2008

Guidelines Committee, American Association of Poison Control Centers. 2003 – 2005.

Terrorism Response Task Force. American College of Emergency Physicians. 2002 – current.

Long Range Planning Committee, American Association of Poison Control Centers. 2000 – current.

Bioterrorism Workgroup on Critical Chemical Agents. Centers for Disease Control and Prevention, 1999.

Federal Legislative Committee, American Association of Poison Control Centers. 1998 - current.

Poison Control Center Leadership Group. Joint Working Group under the Maternal and Child Health Bureau and the National Center for Injury Prevention and Control, Department of Health and Human Services. January 1995 - current.

Invited Participant, "Research Directions in Emergency Medicine", Joint conference of American College of Emergency Physicians, Society of Academic Emergency Medicine and the Emergency Medicine Foundation. Atlanta, Georgia, January 21 - 22, 1995.

Chairman, Research Committee, American College of Emergency Physicians, 1993 - 1995, committee member 1988 - 1995.

Scientific Review Panel, American College of Emergency Physicians, 1993 - 1995.

Scientific Review Committee, American Association of Poison Control Centers, 1990- 2000.

Pharmaceutical and Technology Committee, Society of Academic Emergency Medicine, 1991 - 1992.

COMMITTEES - INTERNATIONAL

Expert Meeting on Envenomations and Their Treatment. World Health Organization. 1995 - current.

MEDICAL SCHOOL TEACHING

Fellowship in Medical Toxicology, approximately 300 hours/year of combined didactic and clinical direct teaching.

Emergency Medicine Residents, 6-1-hour Basic Toxicology lecture per year, 1992 - current.

Internal Medicine Residents Medical Subspecialty Conference at Denver Health Medical Center once per year, 1992 - current.

University of Colorado School of Pharmacy - 4 hr lecture on Clinical Pharmacology of Antidotes to PharmD class per year, 1993 - current.

Lecturer in Introduction to Emergency Medicine Course, University of Colorado, 1993 - current.

PUBLICATIONS

Books

White J, Dart RC. Snakebite: A Brief Medical Guide. Cadmus Communications, Inc. Lancaster, PA. 2007.

GM Bogdan, DL Scherger, S Brady, D Keller, AM Seroka, KM Wruk, J Peterson, DD Swanson, K Ammon, DW Daley, RC Dart, PA Gabow. Health Emergency Assistance Line and Triage Hub (HEALTH) Model. Prepared by Denver Health -- Rocky Mountain Poison and Drug Center under Contract No. 290-0014. AHRQ Publication No. 05-0040. Rockville, MD: Agency for Healthcare Research and Quality. January 2005.

Dart RC. Medical Toxicology 3rd Edition. Lippincott, Williams & Wilkins, Philadelphia, PA, 2004.

Dart RC, Hurlbut KM, Kuffner EK, Yip L (eds). The 5 Minute Toxicology Consult. Lippincott, Williams & Wilkins, Philadelphia, PA 2000.

Peer Reviewed Journals

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LECTURES/PRESENTATIONS

“Use of Data Surveillance”, IMMPACT-X Meeting, Arlington, Virginia, June 3, 2009.

“Monitoring risk: post marketing surveillance and signal detection”. CPDD Meeting. Reno, Nevada, June 24, 2009.

“Toxicity of Cough and Cold Medications”. EAPCCT Annual Meeting. Stockholm, Sweden, May 14, 2009.

“Opioid Abuse, Misuse and Diversion: Strategies for Prevention”. American Academy of Pain Medicine Meeting, Honolulu, Hawaii. January 27, 2009.

“National Stockpile of Medicine for Many Kinds of Threats Including Radiation Event”. Japan Radiation Research Society Annual Meeting. Kitakyushu, Japan, November 21, 2008.

"Introduction to Poison Information Systems and the Rocky Mountain Poison & Drug Center". Bach Mai Hospital, Hanoi, Vietnam, November 2, 2008.

“The Impact of Prescription Opioid Abuse on Young Children (<6 years old): Report of Poison Center Data.” NASCSA Conference. Ft. Lauderdale, FL, October 22, 2008.

“A hiccup in the indictment of cough and cold medicines”. North American Congress of Clinical Toxicology. Toronto, Canada, September 24, 2008.

“Accelerating Research: Integrating Clinical Research with Critical Care”. 44th DIA Annual Meeting. Boston, MA, June 25, 2008.

“Risk Management and Pharmacovigilance for Opioids”. 44th DIA Annual Meeting. Boston, MA, June 25, 2008.

“Simple but Not Easy: New Challenges in Acetaminophen Poisonings”. Emergency Medicine Grand Rounds, Brody School of Medicine, East Carolina University, Greenville, NC, June 11, 2008.

“Antidote Supply and Stocking Issues”. European Association of Poison Centres and Clinical Toxicologists XXVII International Congress. Seville, Spain, May 8, 2008.

“Current Challenges in Buprenorphine Therapy: Monitoring Data and Trends in Data Systems”. American Society of Addiction Medicine’s 39th Annual Medical-Scientific Meeting. Toronto, Canada, April 22, 2008.

“North American Snakebites”. International Toxinology Course. Adelaide, Australia, March 26, 2008.

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“Over the Counter Cough and Cold Medications in Children: Efficacy, Safety and Use”. Society of Toxicology Annual Meeting. Seattle, WA, March 18, 2008.

“Enough is enough! How Long Should we Administer Acetylcysteine? North American Congress of Clinical Toxicology. New Orleans, LA, October 24, 2007.

“Black Widows”. Venom Week, University of Arizona Health Sciences Center. Tucson, AZ, September 7, 2007.

“Consultation and Debriefing Meeting on the Response to Fentanyl-Related Overdoses and Deaths – Lessons for Dealing with future Outbreaks”. U.S. Department of Health and Human

Services Substance Abuse and Mental Health Services Administration. Washington, DC, August 28-29, 2007.

“Methadone Associated Mortality in the RADARS System”, US Department of Health and Human Services. Substance Abuse and Mental Health Services Administration. Center for Substance Abuse Treatment. Washington DC, July 20, 2007.

“New Developments in Acetaminophen Poisoning”. Western Fellow’s Tox Conference, Oakland, CA. April 19, 2007.

“Chronic Pain, Addiction, and the Law: Educational Initiative for Pharmacists in New Mexico”. New Mexico Pharmacists Association 2007 Mid-Winter Meeting. Albuquerque, New Mexico, January 28, 2007.

"Antivenoms - Theory and Practice Overview". North American Congress of Clinical Toxicology, San Francisco, CA, October 8, 2006.

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“The RADARS® System: A National Surveillance System for Misuse and Abuse of Prescription Opioids.” North American Congress of Clinical Toxicology, Orlando, FL, September 11, 2005.

Panel Chair, “Stockpiling and Distribution; Opportunities and Obstacles” panel of “Emergency Medical Preparedness: Obstacles and Opportunities for Coopreration and Collaboration among and within Public and Private Stakeholder Organizations”. Board on Health Sciences Policy, Institute of Medicine, Washington, DC, June 29, 2006.

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“Research Needs in Envenomation: The Treatment is Effective, but the Patient Died.” Reunión Internacional de Expertos en Envenenamiento por Animales Ponzoñosos, Cuernavaca, México. March 18, 2005

“Hepatotoxicity in Alcoholic Patients from Therapeutic Dosing of Acetaminophen.” Society of Toxicology Annual Meeting, New Orleans, LA. March 8, 2005

“Experience with CroFab SERPLINE.” CroFab Symposium, New Orleans, LA. February 10, 2005

"Research Directions in Snake Envenomations." Biology of the Rattlesnakes Symposium, Loma Linda University, Santa Ana, CA. January 17, 2005.

“Management of Acetaminophen Overdose.” Grand Rounds, Vanderbilt University, Nashville, TN, December 13, 2004.

“Use of CroFab in Snake Envenomations.” Grand Rounds, Vanderbilt University, Nashville, TN, December 13, 2004.

“Cyanide Poisoning in Smoke Inhalation and Biochemical Terrorism: Sources, Symptoms and Suggestions.” American College of Emergency Physicians 2004 Scientific Assembly, San Francisco, CA, October 19, 2004.

“New Advances in Treatment of Acetaminophen Poisoning.” Mayo Clinic Department of Emergency Medicine, Rochester, MN, October 5, 2004.

Toxicology Challenges for the Emergency Physician.” Mayo Clinic Department of Emergency Medicine, Rochester, MN, October 5, 2004.

“Ellenhorn Award Lecture.” North American Congress of Clinical Toxicology 2004, Seattle, WA, September 13, 2004.

“WMD Evidence Based Peer Review Protocols for EMS and Hospitals.” 2004 CSEPP National Preparedness Workshop, Seattle, WA, June 15, 2004.

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“WMD Evidence Based Peer Review Protocols for EMS and Hospitals.” 2004 Conference for NDMS (National Disaster Medical System) Participants, Dallas, TX, April 21, 2004.

“Snake Envenomations and Current Management.” 15th Annual Emergency Medicine for the Critically Ill and Injured, Orlando Regional Healthcare, Orlando, FL March 24, 2004.

“Top 10 Toxicology Articles of 2003: What you need to know.” 15th Annual Emergency Medicine for the Critically Ill and Injured, Orlando Regional Healthcare, Orlando, FL, March 24, 2004.

“Toxicology Challenges for the Emergency Patient.” 15th Annual Emergency Medicine for the Critically Ill and Injured, Orlando Regional Healthcare, Orlando, FL March 24, 2004.

“Management of Snake Envenomation” Grand Rounds, Arkansas Children’s Hospital, Little Rock, AR, January 27, 2004.

“North American Snakes & Snakebite.” Clinical Toxinology Training Course, University of Adelaide Faculty of Health Sciences, Adelaide, Australia, September 24, 2003.

“Initial Clinical Experience with Polyvalent Crotalid Antivenin (Ovine) FAB: Do Clinical Trials Reflect Widespread Clinical Use?” IST 14th World Congress on Animal, Plant and Microbial Toxins, Adelaide, Australia, September 19, 2003.

"Intravenous N-Actylcysteine." North American Congress of Clinical Toxicology 2003, Chicago, Illinois, September 7, 2003.

"Implications of FDA Hearings on Acetaminophen, Aspirin, and NSAID Safety, Labeling and Dosing." APhA Self-Care Institute 2003, Charleston, South Carolina, June 21, 2003.

"Evidence Based Approach to the Use of APAP in the Alcoholic Patient." Topics in Emergency Medicine, San Francisco, California, October 21, 2002.

"Experiences of Investigators Looking for Industry Sponsors." Therapies for Rare Disease: From Bench to Marketplace. U.S. Food and Drug Administration, Washington, DC, September 24, 2002.

"Analgesic Use and Alcohol Consumption." Beyond the Headlines: Evaluating the Comparative Risks of OTC Analgesics. Pri-Med 2002, Chicago, Illinois, June 21, 2002.

"Vulnerability to acetaminophen (paracetamol) liver injury during therapeutic dosing: Does it exist?" European Association of Poison Control Centers and Clinical Toxicologists. Lisbon, Portugal. May 2002.

"Snakebite: A Toxicologist's Prospective." 13th Annual Emergency Medicine for the Critically Ill and Injured, Orlando Regional Healthcare, February 15, 2002.

"Acetaminophen Toxicity: New Insights." 13th Annual Emergency Medicine for the Critically Ill and Injured, Orlando Regional Healthcare, February 15, 2002.

"Gut Decontamination. Should We? Really?" 13th Annual Emergency Medicine for the Critically Ill and Injured, Orlando Regional Healthcare, February 15, 2002.

"The Management of Crotaline Snakebite." Emergency Medicine Grand Rounds, University of Alabama, Birmingham, Alabama, August 16' 2001.

"Acetaminophen toxicity: Special issues and update." Quarterly Toxicology Rounds, Henry Ford Hospital, Detroit, Michigan, July 12, 2001.

"Envenomations" Toxicology Has No Borders Conference. University of Texas El Paso. June 22, 2001, El Paso, Texas.

"Evidence based approach to the use of APAP in the ETOH patient." Emergency Medicine. University of Michigan, April 18, 2001, Ann Arbor, Michigan.

"Lessons from the CroFab Trials" Reunion de Expertos en Envenenamiento por Animales Ponzonosos. March 30, 2001, Puebla, Mexico.

"Evidence based approach to the use of APAP in the ETOH patient." Thomas Jefferson University Hospital, November 30, 2000, Philadelphia, Pennsylvania.

"Developing Treatment Guidelines Using Evidence-Based Medicine." North American Congress of Clinical Toxicology, September 15, 2000, Tucson, Arizona.

“Antivenin Vs. Anti-Antivenin.” 27th Annual Rocky Mountain Trauma and Emergency Medicine Conference, July 31, 2000, Steamboat Springs, Colorado.

“Weapons of Mass Destruction: Preparations against Terrorist Acts and Treatment of Victims.” The First Asia-Pacific Regional Conference on Antidote Use and Administration. Foundation for Poison Control, National Poison Center, June 2-3, 2000, Taipei, Taiwan.

“Antivenoms and Dimercapto Chelating Agents.” The First Asia-Pacific Regional Conference on Antidote Use and Administration. Foundation for Poison Control, National Poison Center, June 2-3, 2000, Taipei, Taiwan.

"Clinical Trials of Antibody Therapeutic Agents: Antivenoms and Antidepressants in North America." Department of Pharmacology Special Seminar, University of Melbourne, November 11, 1999, Melbourne, Australia.

"The Role of Paracetamol in the Treatment of the Alcoholic Patient with Pain or Fever - Analysis of the Evidence." Clinical Consensus - An International Update on Paracetamol. November 8, 1999, Sydney, Australia.

"Weapons of Mass Destruction Protocols" National Disaster Medical System Annual Conference. May 11, 1999, Washington, D.C.

"Polyclonal Antibodies as Emergency Therapeutic Agents: Beyond Snakebite" Division of Clinical Pharmacology and Toxicology, Department of Medicine, University of Colorado, May 5, 1999, Denver, Colorado.

"Antibodies as Emergency Therapeutic Agents" Grand Rounds, Internal Medicine, Mayo Foundation Visiting Physician, April 28, 1999, Rochester, Minnesota.

"Advances in Acetaminophen Toxicity" Department of Emergency Medicine, Mayo Foundation Visiting Physician, April 28, 1999, Rochester, Minnesota.

"Therapeutics of Crotalid Snakebite" Grand Rounds, Department of Surgery, Louisiana State University, March 30, 1999, Shreveport, Louisiana

"Antidote Stocking in the United States" Presymposium American Society of Health-System Pharmacists. December 7, 1998, Las Vegas, Nevada.

"Snakebite in United States: Challenges and Opportunities". 92nd Annual Scientific Assembly, Southern Medical Association. November 21, 1998, New Orleans, Louisiana.

"Hot Topics in Toxicology" 92nd Annual Scientific Assembly, Southern Medical Association. November 20, 1998, New Orleans, Louisiana.

"Actions of Poison Centers and Toxicologists in a Chem-Bio Incident: Current Plans and Future Proposals. ACMT Practice Symposium, North American Congress of Clinical Toxicology '98. September 15, 1998, Orlando, Florida.

"Paracetamol: Overdose and Alcohol Experience in the United States." Invited presentation to the Russian Pharmacological Committee. Royal Society of Physicians. June 21, 1998, London, United Kingdom

"Toxic Alcohols" Toxicology Has No Borders. Annual Conference of the Texas Poison Control System. May 2, 1998, El Paso, Texas.

"Venomous Bites" Toxicology Has No Borders. Annual Conference of the Texas Poison Control System. May 2, 1998, El Paso, Texas.

"Treatment Protocols and Pharmaceuticals." 1998 National Conference on Lifesaving Intervention. National Disaster Medical System, United States Public Health Service, March 31, 1998, Denver, Colorado.

"Toxicology Update: New Diseases, New Drugs." Emergency Medicine in the Rural and Suburban Setting. Oklahoma State University. March 1, 1998, Tulsa, Oklahoma.

"Contemporary Issues in the Management of Acetaminophen Overdose." ACEP Pre-conference symposium. October 6, 1997, San Francisco, California.

"Alternative Medicines" Annual Symposium of the American College of Medical Toxicology. September 12, 1997, St. Louis, Missouri.

"Poisons of the Rocky Mountain Region," Annual Meeting, American Society of Forensic Toxicology. October 16, 1996, Denver, Colorado.

"The Cutting Edge: Uncommon Presentations of Common Poisons," North American Congress of Clinical Toxicology. October 11, 1996, Portland, Oregon.

"The Cutting Edge: Snakebite in North America," North American Congress of Clinical Toxicology. October 11, 1996, Portland, Oregon.

"Therapeutic Antibodies: A Revolution in Critical Care Therapeutics?" 17th Annual Practical Therapeutics: A Western Regional Conference. September 22, 1996, Denver Colorado.

"Polyclonal Antibodies: A Revolution in Emergency Medical Therapeutics?" 1996 Annual Meeting, Society of Academic Emergency Medicine, May 5, 1996, Denver, Colorado.

"Poison Control Centers." Colorado Department of Public Health and Environment. August 28, 1995, Denver, Colorado.

"Antibodies as Therapeutic Agents." 5th World Congress of the World Federation of Associations of Clinical Toxicology Centers & Poison Control Centers, November 8-11, 1994, Taipei, Taiwan.

"New Developments in the Treatment of Snake Venom Poisoning." North American Congress of Clinical Toxicology. 1994 AAPCC/AACT/ACMT/CAPCC Scientific Meeting. September 23-26, 1994, Salt Lake City, Utah.

"The Management of Crotalid Snakebite" Pediatric Grand Rounds, Presbyterian/St. Luke's Medical Center, August 5, 1994, Denver, Colorado.

"Polyclonal Antibodies and Therapeutic Agents." Visiting Professorship, Maricopa Medical Center, June 22, 1994, Phoenix, Arizona.

"Advances in the Treatment of Snake Venom Poisoning." International Herpetological Society - Annual Meeting, June 17, 1994, New Orleans, Louisiana.

"Polyclonal Antibodies: An Emerging Therapy for Venomous and Pharmacologic Poisoning." Emergency Medicine for the Critically Ill and Injured. Orlando, Florida, February 26, 1994.

"Gastrointestinal Decontamination in Toxic Ingestions: A Daily Decision for Emergency Physicians." Emergency Medicine for the Critically Ill and Injured. Orlando, Florida, February 26, 1994.

"Polyclonal Antibodies: A Revolution in Emergency Medical Therapeutics?" Emergency Medicine Grand Rounds, University of Colorado, February 2, 1994.

"Critical Concepts in the Management of Acute Iron Poisoning in Children", Denver Children's Hospital, Denver, Colorado, August 2, 1993.

"Medical Management of North American Snake Venom Poisoning", Veterans General Hospital, Taipei, Taiwan, December 10, 1992.

"Clinical Toxicology of Lead", Human Exposure to Lead and Toxic Chemicals, Taiwan National Institute of Occupational Safety and Veterans General Hospital, Taipei, Taiwan, December 9, 1992.

"Clinical Aspects and Management of Metal and Toxic Exposure", Human Exposure to Lead and Toxic Chemicals, Taiwan National Institute of Occupational Safety and Veterans General Hospital, Taipei, Taiwan, December 8, 1992.

"Critical Decisions in the Management of Snake Venom Poisoning" Department of Surgery, Section of Emergency Medicine Grand Rounds, University of Arizona Health Sciences Center, July 21, 1992.

"Necrotic Arachnidism" Department of Medicine, Veterans Administration Medical Center, Tucson, Arizona, April 9, 1992.

"Clinical Toxicology and Treatment of Drug Overdose" RW Bliss Army Community Hospital, Fort Huachuca, Arizona, February 19, 1992.

"Rational Use of Antidotes" Clinical Pharmacology/Toxicology Lecture Series. Arizona Health Science Center, Tucson, Arizona, January 15, 1992.

"New Advances in Toxicology" Grand Rounds, Section of Emergency Medicine. Arizona Health Sciences Center, Tucson, Arizona, March 10, 1991.

"Antibodies as Therapeutic Agents" Department of Surgery Grand Rounds, University of Arizona, February 12, 1992.

"The Unknown Bite" Division of Emergency Medicine, University of Colorado, Denver, CO, January 29, 1992.

"Snakes, Scorpions, Spiders" Southwestern Poison Symposium, Tucson, Arizona, November 9, 1991.

"Toxicity of Dental Amalgams" Southwestern Poison Symposium, Tucson, Arizona, November 8, 1991.

"Southwestern Envenomations" Southwestern Trauma Symposium, Tucson Arizona, August 19, 1991.

"Bites and Stings" Grand Rounds, Section of Emergency Medicine. Arizona Health Sciences Center, Tucson, Arizona, July 9, 1991.

"Envenomations by the Brown Spider" Section of Dermatology, Arizona Health Sciences Center, Tucson, Arizona, May 29, 1991.

"Importance of Oxygen Free Radicals to the Surgeon" Grand Rounds, Department of Surgery, Arizona Health Sciences Center, Tucson, Arizona, October 13, 1990.

Moderator, "Metal Toxicology Workshop." AAPCC/ABMT/AACT Annual Scientific Symposium, Tucson, Arizona, September 16, 1990.

"Arthropod Envenomations." University of California at San Diego Conference: Wilderness Emergencies. Snowmass, Colorado, August 17, 1990.

"Role of Oxygen Radicals in Hemorrhagic Shock - Resuscitation." Division of Surgical Biology, Department of Surgery, Arizona Health Sciences Center, Tucson, Arizona, July 17, 1990

"Principles of Clinical Toxicology." Grand Rounds, Section of Emergency Medicine. Arizona Health Sciences Center, Tucson, Arizona, July 17, 1990.

"Role of Oxygen Radicals in Hemorrhagic Shock." Surgical Biology Seminar. Surgical Biology staff and students. May 15, 1990.

"Role of Fasciotomy in the Management of Pit Viper Poisoning." Darnall Army Base Emergency Medicine Residency. May 30, 1990. Killeen, Texas.

"Southwestern Envenomations." UCLA Emergency Medicine Trauma Conference. May 31, 1990. Los Angeles, California.

"On the Horizon." American College of Emergency Physicians. Winter Symposium, Tucson, Arizona. March 15, 1990.

"Sequelae of Pit Viper Envenomation". Symposium on Biology of Pit Vipers. Texas Herpetological Association. November 19, 1989. Arlington, Texas.

"Pediatric Envenomations". Ninth Annual Southwestern Poison and Drug Information Symposium. November 9, 1989. Tucson, Arizona.

"Venomous Bites of Southern Arizona". Southwest Arizona Area Health Education Center, Nogales, Arizona, December 9, 1989.

"Management of Pediatric Envenomation." Grand Rounds. Section of Emergency Medicine, Arizona Health Sciences Center. Tucson, Arizona, September 1989.

"Management of Poisonous Snakebite in Children." Grand Rounds. Dept. of Pediatrics, Tucson, Arizona, July 1989.

"Hemorrhagic Shock." Emergency Medicine Grand Rounds
University of Arizona, Tucson, Arizona, 1988.

"Update on Antidotes." Arizona Society of Hospital Pharmacists, Tucson, Arizona, November 1988.

"Chelation Therapy." Seventh Annual Southwestern Poison and Drug Information Symposium, Tucson, Arizona, November 14, 1987

"Reperfusion Injury After Hemorrhagic Shock." Pharmacology Graduate Student Seminar. Tucson, Arizona, October 14, 1987

"Rattlesnake Envenomation: Role of Fasciotomy." Surgical Grand Rounds, Tucson, Arizona, September 12, 1987

"Rattlesnake Envenomation." Arizona Medical Association
Annual Scientific Meeting Phoenix, Arizona, June 4, 1987

"Controversies in Emergency Medicine: Snakebite." Emergency Medicine Grand Rounds, Arizona Health Sciences Center, Tucson, Arizona, October 19, 1986.

"Radiation Emergencies: Emergency Medical Response to Hazardous and Toxic Material Exposures." University of Arizona, Tucson, Arizona, February 1986.

"Development of Research Ideas." Emergency Medicine Grand Rounds, Arizona Health Sciences Center, Tucson, Arizona, January 1986

"Update on Snake Venom Poisoning." California Medical Center Hospital, Los Angeles, October 1985

RESEARCH FUNDING

PI: Dart RC, Bogdan GM
Project: Antidote Summit: literature and national consensus panel stocking recommendations
Source: Multiple
Period: 2007-2008

PI: Dart RC, Green JL
Project: Evaluation of pediatric exposures to OTC oral cough & cold products
Source: Multiple
Period: 2007-2009

PI: Dart RC
Project: The RADARS® System
Source: Multiple
Period: 2006-2009

PI: Dart RC
Project: Phase II and III trials of black widow spider antivenom
Source: Rare Disease Therapeutics
Period: 2005-2010

PI: Dart RC, Heard KF, Green JL
Project: Aminotransferase trends during prolonged therapeutic dosing with acetaminophen
Source: McNeil Consumer Specialty Products
Period: 2008-2009

PI: Dart RC, Heard KF, Green JL
Project: Prospective trial of therapeutic acetaminophen use in non-drinking patients
Source: McNeil Consumer Specialty Products
Period: 2005-2008

PI: Dart RC, Heard KF, Green JL
Project: Utility of acetaminophen-protein adducts in the determination of acetaminophen exposures
Source: McNeil Consumer Specialty Products
Period: 2005-2009

PI: Dart RC
Project: Toxicology Investigators Network (ToxIN) - Retrospective comparison of safety and efficacy between the oral and intravenous forms of N-acetylcysteine.
Source: American Academy of Clinical Toxicology & Cumberland Pharmaceuticals
Period: 2005-2008

PI: Dart RC
Project: Meta-Analysis of intravenous N-acetylcysteine efficacy and safety.
Source: Cumberland Pharmaceuticals
Period: 2001-2004

PI: Dart RC
Project: Development of a multi-center clinical protocol for a prospective trial of therapeutic acetaminophen use in alcoholic patients.
Source: McNeil Consumer Specialty Products
Period: 2001-2004

PI: Dart RC

Project: Development of a clinical protocol for a prospective trial of therapeutic acetaminophen use in moderate drinkers.
Source: McNeil Consumer Specialty Products
Period: 2003-2004

PI: Dart RC
Project: Coordinate and conduct a multi-center surveillance project on opioid abuse reported to poison centers.
Source: PurduPharma Inc.
Period: 2002-2005

PI: Anderson MA, Bogdan GM, Dart RC
Project: Develop a Pediatric Environmental Health Specialty Unit (PEHSU) for US Region 8
Source: Association of Occupational & Environmental Clinics (AOEC)
Period: 2001-2009

PI: Anderson MA, Bogdan GM, Dart RC
Project: Provide outreach to Colorado physicians about elevated blood lead in children
Source: Colorado Department of Public Health & Environment (CDPHE)
Period: 2003

PI: Bronstein AC, Dart RC
Project: Pilot project to analyze utility of poison center data as part of real-time toxicosurveillance system in Colorado
Source: Colorado Department of Public Health & Environment (CDPHE)
Period: 2003-2004

PI: Davidson A, Dart RC, Bogdan GM
Project: Denver Center for Advanced Public Health Preparedness
Source: Centers for Disease Control and Prevention – Health Alert Network Exemplar Grant
Period: 1999-2003

PI: Dart RC
Project: Treatment of acetaminophen overdose with intravenous N-Acetylcysteine
Source: Cumberland Pharmaceuticals, Inc
Period: 2000-2001

PI: Dart RC, Bogdan GM
Project: Development of a Denver Metropolitan Medical Response System
Source: US Department of Health & Human Services – US Public Health Service
Period: 1997-2001

PI: Dart RC
Project: Analysis & Update of the 1996 Olympic Clinical Treatment Protocols for Casualties Resulting from Terrorist Incidents Involving Weapons of Mass Destruction by Consensus Review
Source: US Department of Health & Human Services – Office of Emergency Preparedness
Period: 1998-2000

PI: Daly FR, Dart RC

Project: Neutralization of *Latrodectus mactans* venom by antivenom developed for *Latrodectus hasseltii* in humans, a prospective case series
Source: Rocky Mountain Poison & Drug Foundation
Period: 1998-2000

PI: Daly FR, Dart RC
Project: Neutralization of *Latrodectus mactans* venom by antivenom developed for *Latrodectus hasseltii*
Source: Rocky Mountain Poison & Drug Foundation
Period: 1998-2000

PI: Dart RC
Project: Controlled clinical trial of the accuracy of dimercaptopropane sodium sulfonate (DMPS) as a diagnostic agent for mercury and arsenic body burden
Source: Heyl Chemisch-Pharm
Period: 1998-2000

PI: Waksman JC, Dart RC
Project: Study of tumor necrosis factor levels resulting from a variety of patient ingestions
Source: Rocky Mountain Poison & Drug Foundation
Period: 1998-2000

PI: Bogdan GM, Dart RC
Project: Rocky Mountain Arsenal Medical Monitoring Program - Referral System
Source: Colorado Department of Public Health & Environment
Period: 1997-2008

PI: Dart RC
Project: Trial to determine the safety, pharmacokinetics and pharmacodynamics of affinity purified digoxin immune Fab (ovine, lyophilized)
Source: Protherics, Inc. (formerly Therapeutic Antibodies, Inc.)
Period: 1996-2000

PI: Dart RC
Project: Surveillance of pediatric blood lead levels reported to Rocky Mountain Poison & Drug Center.
Source: Colorado Department of Public Health & Environment (CDPHE)
Period: 1995-2000

PI: Dart RC
Project: Comparison of reconstitution times for CroFab and Antivenin (Crotalidae) Polyvalent [Wyeth]
Source: Savage Laboratories - Altana Inc.
Period: 1999

PI: Dart RC
Project: Revision of Overdose Sections of Package Inserts
Source: Schering Corporation
Period: 1999

PI: Dart RC, Bogdan GM
Project: Impact of a Poison Center on Insufficient Stocking of Antidotes
Source: Orphan Medical Inc
Period: 1998-1999

PI: Dart RC
Project: Combined evidence-based literature analysis and consensus guidelines for stocking of emergency antidotes in the United States
Source: US Department of Health & Human Services - Health Resources and Services Administration
Period: 1998-1999

PI: Dart RC, O'Malley GF
Project: Development of a model for crotalid venom-induced histopathologic changes in mammalian joints
Source: University of Colorado Health Sciences Center Department of Surgery Dean's Grant
Period: 1998-1999

PI: Heard K, Dart RC
Project: Geriatric Poisoning - Epidemiology and Outcome of Elderly Poisoning Patients
Source: Society of Academic Emergency Medicine, CORD Geriatric Emergency Medicine Resident/Fellow Grant
Period: 1998-1999

PI: Dart RC, Bogdan GM
Project: Registry to record the adverse effects and development of recurrence after typical clinical use of Antivenin (Crotalidae) Polyvalent [Wyeth]
Source: Protherics, Inc.
Period: 1997-1999

PI: Aposhian HV, Dart RC
Project: DMSA, DMPS, and other Orally Active Dithiol Chelators
Source: National Institutes of Environmental Health & Safety
Period: 1992-2000

PI: Dart RC
Project: An Open-Label Safety Study of An Escalating Dosage Regimen of Affinity Purified Ovine Broad Spectrum Cyclic Antidepressant Immune Fab (TriTAb)
Source: Protherics, Inc. (formerly Therapeutic Antibodies, Inc.)
Period: 1997-1999

PI: Dart RC
Project: Development of verapamil-specific polyclonal antibody Fab fragments to reverse verapamil cardiotoxicity
Source: American Academy of Clinical Toxicology & Rocky Mountain Poison & Drug Foundation
Period: 1997-1999

PI: Dart RC, Bogdan GM
Project: Clinical Trial of a New Antidote for Ethylene Glycol & Methanol Intoxication

Source: Orphan Medical, Inc.
Period: 1997-1999

PI: Dart RC, Kuffner EK
Project: Evaluation of hepatotoxicity in alcoholics from therapeutic dosing of acetaminophen
Source: McNeil Consumer Products Inc.
Period: 1996-1999

PI: Dart RC
Project: Trial to determine the safety, pharmacokinetics and pharmacodynamics of affinity purified digoxin immune Fab (ovine, lyophilized)
Source: Protherics, Inc. (formerly Therapeutic Antibodies, Inc.)
Period: 1996-1999

PI: Dart RC, Bogdan GM
Project: Study to develop a registry of human exposures to *N,N*-Diethyl-*m*-toluamide (DEET)
Source: Chemical Specialty Manufacturers Association
Period: 1995-1999

PI: Dart RC
Project: Treatment of acetaminophen overdose with intravenous N-Acetylcysteine
Source: Apothecon Inc.
Period: 1995-1999

PI: Dart RC
Project: An Open-Label, Parallel Comparative Study Between Two Dosing Regimens of CroTAb™ affinity purified immune Fab (ovine, lyophilized)
Source: Protherics, Inc. (formerly Therapeutic Antibodies, Inc.)
Period: 1997-1998

PI: Dart RC
Project: Unrestricted Educational Grant
Source: Williams and Wilkins Publishing
Period: 1997

PI: Dart RC, Denzel D
Project: Therapeutic Guidelines on Surgical and Non-Surgical Antimicrobial Prophylaxis
Source: American Society of Health-System Pharmacists
Period: 1995-1997

PI: Palmer ME, Dart RC (preceptor)
Project: Snakebite Antivenin Trials in Plants from Belize: Bioassay screen, dose response relationships, and cross-reactivity among two venoms
Source: Emergency Medicine Foundation
Period: 1995-1996

PI: Dart RC, Decker W
Project: Stability of Crotalidae Polyvalent Antivenom under field conditions
Source: Wilderness Medical Society
Period: 1994-1995

PI: Dart RC
Project: Efficacy and pharmacokinetics of affinity purified digoxin immune Fab (ovine, lyophilized) in a rat model
Source: Therapeutic Antibodies, Inc
Period: 1993-1995

PI: Dart RC, Hurlbut KM
Project: Development of a severity score for the assessment of poisonous snakebite
Source: Therapeutic Antibodies, Inc
Period: 1993-1994

PI: Dart RC
Project: Analysis of 4-tert-amylphenol and 2-phenylphenol toxicity.
Source: Genetics Institute, Inc
Period: 1998

PI: Dart RC.
Project: A clinical trial of affinity purified immune Fab (ovine, lyophilized) antivenom.
Source: US Food & Drug Administration Orphan Drug Grant
Period: 1993-1995

PI: Dart RC.
Project: Isolation and efficacy of Anti-Scorpion Fab.
Source: Arizona Disease Control Research Commission
Period: 1992-1993

PI: Bernstein JN, Dart RC
Project: Development of a Fab Scorpion Antivenom.
Source: Emergency Medicine Foundation.
Period: 1992-1993

PI: Dart RC, Burgess JB
Project: The effect of constriction bands on rattlesnake venom absorption: A pharmacokinetic study.
Source: Emergency Medicine Foundation
Period: 1990

PI: Sullivan JB, Dart RC:
Project: Testing of anti-tricyclic F(ab) fragments in a porcine model.
Source: Burroughs Wellcome
Period: 1990-1991

PI: Dart RC, Meislin HW
Project: Evaluation of ketorolac tromethamine IM and meperidine hydrochloride IM in patients with pain in a hospital emergency room.
Source: Syntex Laboratories, Inc.
Period: 1990-1991

PI: Dart RC

Project: The safety and efficacy of temafloxacin 600 mg BID compared to cefadroxil 500 mg BID in the treatment of bacterial skin or skin structure infection.
Source: Roche Pharmaceutical
Period: 1989

PI: Dart RC
Project: Role of Oxygen Radical Generated Lipid Peroxidation in Resuscitation From Cardiac Arrest.
Source American Heart Association, Arizona Affiliate
Period: 1988

PI: Dart RC
Project: Delivery of Rattlesnake Antivenin Across The Skin.
Source: Arizona Disease Control Research Commission.
Period: 1985-1986

6/9/2010

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
08/405,454	03/15/1995	JOHN B. SULLIVAN	P0786.70000US05	6004

7590 06/15/2009

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EXAMINER	
SCHWADRON, RONALD B	
ART UNIT	PAPER NUMBER
1644	

MAIL DATE	DELIVERY MODE
06/15/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

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Wolf, Greenfield & Sacks, P.C.

JUN 17 2009

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Wolf, Greenfield & Sacks, P.C.

JUN 17 2009

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MTJ

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

Ex parte JOHN B. SULLIVAN and FINDLAY E. RUSSELL

Appeal 2009-002479
Application 08/405,454
Technology Center 1600

Decided:[1] June 15, 2009

Before MICHAEL R. FLEMING, *Chief Administrative Patent Judge,* JAMES T. MOORE, *Vice Chief Administrative Patent Judge,* and DONALD E. ADAMS, DEMETRA J. MILLS, SALLY G. LANE, ERIC GRIMES, and LORA M. GREEN, *Administrative Patent Judges.*

ADAMS, *Administrative Patent Judge.*

DECISION ON APPEAL

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[1] The two-month time period for filing an appeal or commencing a civil action, as recited in 37 C.F.R. § 1.304, begins to run from the decided date shown on this page of the decision. The time period does not run from the Mail Date (paper delivery) or Notification Date (electronic delivery).

This appeal under 35 U.S.C. § 134 involves claims 40-42 and 50. The only remaining pending claims, claims 54 and 55, were withdrawn from consideration as drawn to a non-elected invention (*see e.g.*, App. Br. 1). We have jurisdiction under 35 U.S.C. § 6(b).

STATEMENT OF THE CASE

This Appeal is back before the Board on remand from the Court of Appeals for the Federal Circuit, our appellate reviewing court.

The Federal Circuit found that "a prima facie case of obviousness was established [in this case] because Sullivan teaches whole antibodies for use against rattlesnake venom and Coulter teaches using Fab fragments to detect venom of a different snake." *In re Sullivan*, 498 F.3d 1345, 1351 (Fed. Cir. 2007).

Nevertheless, our appellate reviewing court found that the Board failed to give sufficient weight to Appellants' declaratory evidence. Specifically, it found that the following three pieces of evidence in the record required further consideration: (1) the Smith Declaration, which "is relevant as evidence that the prior art taught away from the claimed invention"; (2) the Sullivan Declaration, which "describes an unexpected property or result from the use of Fab fragment antivenom"; and (3) the First Russell Declaration, which "discusses why those having ordinary skill in the art expected antivenoms comprising Fab fragments to fail." *Id.* at 1352.

Accordingly, the Federal Circuit vacated the Board's March 30, 2006 Decision affirming the rejection of claims 40-42 and 50 under 35 U.S.C. § 103 as being unpatentable over the combination of Sullivan and Coulter. *See id.* at 1352-53. Therefore, we reconsider the record before us on appeal *de novo*, carefully evaluating and weighing both the evidence relied upon by

the Examiner and the objective evidence of nonobviousness provided by Appellants.

The claims are directed to an antivenom pharmaceutical composition. Claim 40 is illustrative of the subject matter on appeal and is reproduced below:

> 40. An antivenom pharmaceutical composition for treating a snakebite victim, comprising Fab fragments which bind specifically to a venom of a snake of the Crotalus genus and which are essentially free from contaminating Fc as determined by immunoelectrophoresis using anti-Fc antibodies, and a pharmaceutically acceptable carrier, wherein said antivenom pharmaceutical composition neutralizes the lethality of the venom of a snake of the Crotalus genus.

The Examiner relies on the following evidence:

Stedman's Medical Dictionary 94 (23rd ed., Williams and Wilkins Co., 1976).

Smith et al., *Immunogenicity and kinetics of distribution and elimination of sheep digoxin-specific IgG and Fab fragments in the rabbit and baboon,* 36 CLIN. EXP. IMMUNOL. 384-96 (1979).

J. B. Sullivan, Jr. and F. E. Russell, *ISOLATION AND PURIFICATION OF ANTIBODIES TO RATTLESNAKE VENOM BY AFFINITY CHROMATOGRAPHY,* 25 PROC. WEST. PHARMACOL. SOC. 185-92 (1982).

Alan Coulter and Rodney Harris, *Simplified Preparation of Rabbit Fab Fragments*, 59 J. IMMUNOL. METHODS 199-203 (1983).

Appellants rely on the following evidence:

First Russell Declaration, executed April 30, 1998.

Smith Declaration, executed April 24, 1995.

Sullivan Declaration, executed September 25, 1995.

Stewart Sell, M.D., *Basic Immunology: Immune Mechanisms in Health and Disease,* 89 (ed., Elsevier, New York, NY) (1957).

Findlay E. Russell, M.D., Ph.D., *Snake Venom Poisoning* 5, 139, and 168 (ed., J.B. Lippincott Co., Philadelphia, PA) (1980).

Faulstich et al., *STRONGLY ENHANCED TOXICITY OF THE MUSHROOM TOXIN α-AMATOXIN BY AN AMATOXIN-SPECIFIC FAB OR MONOCLONAL ANTIBODY,* 26 TOXICON 491-499 (1988).

Russell, *SNAKE VENOM IMMUNOLOGY: HISTORICAL AND PRACTICAL CONSIDERATIONS,* 7 J. TOXICOL. - TOXIN REVIEWS 1-82 (1988).

Joseph Balthasar and Ho-Leung Fung, *Utilization of Antidrug Antibody Fragments for the Optimization of Intraperitoneal Drug Therapy: Studies Using Digoxin as a Model Drug,* 268 J. PHARM. EXP. THER. 734-739 (1994).

Sorkine et al., *Comparison of $F(ab')_2$ and Fab efficiency on plasma extravasation induced by Viper aspis venom*, 33 TOXICON 257 (1995).

Ownby et. al., *Levels of Therapeutic Antivenin and Venom in a Human Snakebite Victim*, 89 SOUTHERN MEDICAL JOURNAL 803-807 (1996).

Russell, *Toxic Effects of Animal Toxins,* in *Casarett and Doull's Toxicology: The Basic Science of Poisons* 801-805 (5th ed., McGraw-Hill, New York, NY) (1996).

WHO Coordination Meeting on Venoms and Antivenoms, WHO/B5/80-1292 BLG/Ven/80.1 Rev. 1 (Date unknown).

The rejections presented by the Examiner are as follows:

1. Claims 40-42 and 50 stand rejected under 35 U.S.C. § 103 as being unpatentable over Sullivan in view of Coulter.
2. Claims 40-42 and 50 stand rejected under 35 U.S.C. § 103 as being unpatentable over Sullivan in view of Coulter, Smith and Stedman's.

We affirm.

The combination of Sullivan in view of Coulter:

ISSUES

Given that the Federal Circuit held that a prima facie case of obviousness has been established on this record, the issue before us distills down to whether the prima facie case of obviousness over the combination of Sullivan and Coulter stands when reconsidered in view of Appellants' arguments and Declaratory evidence on this record.

FINDINGS OF FACT

FF 1. The instant application has an effective filing date of October 9, 1984.

FF 2. "A venom is a toxic substance produced by a plant or animal . . . and usually delivered through a biting or stinging act" (First Russell Declaration 3: ¶ 15). "Antivenin is a suspension of venom-neutralizing antibodies prepared from the serum of animals . . . hyperimmunized against a specific venom or venoms" (Spec. 4: 19-22; First Russell Declaration 5: ¶ 18). "[T]he terms 'antivenin' and 'antivenom' are now interchangeable" in the art (First Russell Declaration 5: ¶ 18).

FF 3. Russell declares that during envenomation, "[v]enom components are usually injected into subcutaneous tissues. Since many of the venom toxins are large, hydrophobic molecules, they are slowly released from these injection areas. This results in the 'venom depot effect' where toxins are continuously released into the systemic circulation long after the initial bite" (First Russell Declaration 9: ¶ 30).

FF 4. Russell declares that "[S]nake venoms of the family Crotalidae comprise at least 20 different compounds. In some *Crotalus sp.* snake

venoms, there may be 100 different protein fractions, 25 of which may be enzymes. Due to their complexity, the full composition of snake venoms is unknown" and "the pharmacological effects of some constituent toxins are unknown" (First Russell Declaration 4: ¶ 15-16).

FF 5. Russell declares that "[i]mmunoglobulins neutralize toxins in several ways. For example, they bind specifically to epitopes present on the toxins. In the case of a polyclonal antivenom, this may involve several epitopes present on more than one antigen" (First Russell Declaration 8-9: ¶ 28).

FF 6. At the time the invention was made the "therapeutic modality for treatment of Crotalidae envenomation [in humans] in the United States involves the intravenous administration of equine source Antivenin (Crotalidae) Polyvalent (ACP)" (Sullivan 185: 1-3; *see also* First Russell Declaration 5-6: ¶ 19 and 6: ¶ 21).

FF 7. Smith declares that "Antivenins comprising intact antibodies have been sold commercially since at least 1947. Antivenins comprising $F(ab)_2$ fragments have been sold commercially since at least 1969." (Smith Declaration 2: ¶ 7). Russell declares that "At the time of the application, the only commercially available antivenom for envenomation by North American snakes of the family Crotalidae was Antivenin (Crotalidae) Polyvalent [(ACP)] (equine origin) (Wyeth Laboratories, Philadelphia, PA)" (First Russell Declaration 5-6: ¶ 19).

FF 8. Sullivan teaches "purified antivenin polyvalent antibodies derived from horse hyperimmune antisera against venom of the Crotalus genus [(ACP)] (see Methods section, pages 185-187)" (Ans. 5). Sullivan teaches that the ACP antibodies neutralize the lethality of the venom of a snake of the Crotalus genus (Sullivan 187: 18-23).

FF 9. Russell declares that "[s]oon after the development of the first antivenoms, doctors recognized that they could elicit serum sickness, an allergic reaction to the antisera that was sometimes more deleterious than the venom. Over 75% of patients treated with ACP develop some manifestation of serum sickness" (First Russell Declaration 6: ¶ 21; *see also* Sullivan 185: 3-8).

FF 10. Sullivan teaches "that reducing the immunogenicity of polyvalent horse antivenin is an important goal, due to immune reactions that limit the clinical efficacy of antivenin preparations which contain only partially purified hyperimmune horse antisera (see page 185, first paragraph)" (Ans. 7). In this regard, Sullivan teaches that the incidence of serum sickness reactions should be significantly reduced by the removal of extraneous foreign protein (Sullivan 190: 9 - 191: 2).

FF 11. Russell declares that because "serum sickness results from immune reactions of the patient to the immunoglobulin component of the antivenom, which actually binds to the venom toxins, . . . research focused on using fragments of immunoglobulin molecules that might not provoke a[n] immune reaction" (First Russell Declaration 7: ¶ 22; *see also* Spec. 2: 38 - 3: 2 ("It is also well known in the art that the smaller F(ab) fragments are less likely to cause undesired immunogenic reactions. A general rule is that, given possession of the antibody active site, the smaller the antibody molecule the better")).

FF 12. "Sullivan does not teach a F(ab) containing antivenin" (Ans. 5). According to Russell, "[d]espite known problems with the only commercially available antisera for Crotalidae envenomation and much research since 1947, no researcher had developed an antivenom comprising

Fab fragments" (First Russell Declaration 14: ¶ 43). In this regard, Russell declares that "those of ordinary skill in the art had not progressed beyond $F(ab)_2$ fragments to the smaller Fab fragments" (*id.*). *See also*, Sullivan Declaration 3: ¶ 5 ("The development of antivenin production through the years stopped at a final product of $F(ab)_2$'s").

FF 13. Coulter teaches an anti-Australian brown snake toxin composition comprising Fab fragments that are free of Fc in a pharmaceutically acceptable carrier (PBS) (Coulter 200: 10-23; Ans. 6). Coulter teaches a method for preparing rabbit-derived Fab fragments that includes the removal of undigested immunoglobulin and Fc fragments (Coulter 200: 19-20).

FF 14. The toxin used in Coulter's study, textilotoxin, is a single toxin from the venom of the Australian brown snake (*Pseudonaja textilis*), which is not a member of the genus *Crotalidae* (First Russell Declaration 15: ¶ 46 and ¶ 47).

FF 15. Coulter teaches the use of Fab fragments to detect venom of a snake. (Coulter 201: 1-15; 202: 7-12). In this regard, Coulter teaches that higher assay sensitivity has been observed when Fab is used instead of intact IgG in immunoassays (Coulter 199: 2-3).

FF 16. Coulter teaches that a composition comprising F(ab) fragments reactive against a snake toxin is capable of neutralizing the lethality of that snake toxin in vivo (Ans. 7).

FF 17. Russell declares that at the time the invention was made, persons of ordinary skill in this art recognized that due to their small size, Fab fragments can be distributed to more parts of the body than the larger $F(ab')_2$ and intact IgG molecules (First Russell Declaration 11: ¶ 35; *see also* Spec.

21: 6-8; and Sorkine 257: 13-14 (The smaller size of Fab relative to $F(ab)_2$ results in faster diffusion and a greater volume of distribution)).

FF 18. In addition, and consistent with the statements of Russell, Smith, and Sullivan, at the time this invention was made, persons of ordinary skill in this art recognized that "Fab fragments are small enough to be removed by the renal system. Consequently they have a half-life of about 17 hours" and "are completely eliminated in only 24 to 26 hours" (First Russell Declaration 9-10: ¶ 31; Sullivan Declaration 3: ¶ 5). However, F(ab) fragments in complex with venom protein are "too large to be excreted rapidly by glomerular filtration" (Smith Declaration 2: ¶ 6; Sullivan Declaration 3: ¶ 5). The same is true of "$F(ab)_2$ fragments and whole IgG[, which] are also too large to be eliminated by the renal system . . . [and therefore] have a longer half-life, approximately 50 hours," relative to [uncomplexed] Fab fragments (First Russell Declaration 10: ¶ 32; *see also* Sullivan Declaration 5: ¶ 5).

FF 19. Faulstich teaches that "[t]oxicity in mice of α-amanitin (i.p.), followed by i.v. administration of a monoclonal antibody, is very similar to the toxicity caused by i.v. administration of the amanitin-immunoglobulin complex" (Faulstich 495: 6-8). Similarly, in Coulter's study, textilotoxin was first mixed with Fab fragments *in vitro* and then the Fab-textilotoxin complex was injected intravenously. Russell declares that "[t]his treatment with Fab fragments resulted in neutralization that was essentially equivalent to the treatment with the IgG fragments, just as one would have expected" (First Russell Declaration 16: ¶ 48). Sorkine conducted a similar experiment wherein Fab fragments were mixed with a venom of a non-Crotalidae snake prior to injection into a mouse, and they obtained results similar to those observed by Coulter (First Russell Declaration 17: ¶ 50). Sullivan also

teaches a study wherein venom and antibody are mixed prior to injection into an animal to establish the antibody's ability to protect against lethality of the venom (Sullivan 187: 18-23). Appellants also determine lethality of the toxin by injecting a complex of toxin and immunoglobulin, or fragment thereof, into an animal (Spec. 18-19).

FF 20. After Appellants' effective filing date Faulstich identified an intact antibody and its corresponding Fab fragment that were both incapable of neutralizing the toxicity of the mushroom toxin (α-amatoxin) (Faulstich 497: 18-23).

FF 21. Faulstich reports that not only were the Fab fragments unable to neutralize the toxicity of α-amatoxin in mice, but they increased the toxicity of α-amatoxin by a factor of 50 (First Russell Declaration 12: ¶ 38). According to the post-filing date Faulstich reference "[t]o our knowledge this is the first reported case where immunoglobulins or their fragments enhance rather than decrease the activity of a toxin" (Faulstich 491: Abstract). The post-filing date Balthasar reference cites Faulstich's work and states that "[t]he risk of redistributing systemic toxicity, rather than minimizing systemic toxicity, should be appreciated as a potential outcome" of the use of antibodies to neutralize the toxicity of a toxin (Balthasar 738: col. 2, ll. 3-6; First Russell Declaration 13: ¶ 40).

FF 22. Sorkine reports that when antibody fragments are not mixed with venom prior to injection a larger concentration of antibody fragments is necessary to neutralize the toxin, "Fab being five times more effective than $F(ab')_2$" (Sorkine). In addition, Sorkine's

> data showed firstly that the *in vitro* neutralization of the venom by immunoglobulin fragments does not reflect their *in vivo* efficiency. Secondly, Fab was considerably more effective than

> $F(ab')_2$ in reducing CPI [capillary permeability increase] induced by venom. One explanation is the different kinetics of these fragments. The smaller size of Fab results in faster diffusion and a greater volume of distribution.

(Sorkine 257: 11-14.)

FF 23. Smith discusses a post-filing date clinical study of the treatment of *Vipera berus* envenomation with the purified ovine F(ab) fragment TAb001 and "conventional" $F(ab)_2$ antivenom (Smith Declaration 2: ¶ 11-13). Smith declares that

> TAb001 appears to be equally effective as the conventional antivenom in reducing the occurrence of extensive edema and severe anemia as well as shortening hospital stay. Moreover, to date, no allergic events, suggesting an immediate or delayed hypersensitivity response, have been observed after administration of TAb001, whereas 10% of those given conventional antivenom had allergic side-effects.

(Smith Declaration 5-6: ¶ 13.)

FF 24. Appellants' Specification discloses a comparison of the ability of intact ACP antibody and Fab fragments to protect against snake venom (Spec. 18-23). Appellants disclose that while the dosage "will be adjusted to suit the particular circumstances of the envenomation" the data indicates that both F(ab) fragments and intact IgG "can be used in the treatment of human snake bite victims" (Spec. 23).

PRINCIPLES OF LAW

The question of obviousness is resolved on the basis of underlying factual determinations including: (1) the scope and content of the prior art; (2) the level of ordinary skill in the art; (3) the differences between the claimed invention and the prior art; and (4) secondary considerations of

nonobviousness, if any. *Graham v. John Deere Co.*, 383 U.S. 1, 17-18 (1966).

"[D]uring examination proceedings, claims are given their broadest reasonable interpretation consistent with the specification." *In re Hyatt*, 211 F.3d 1367, 1372 (Fed. Cir. 2000). "[A] claim preamble has the import that the claim as a whole suggests for it." *Bell Communications Research, Inc. v. Vitalink Communications Corp.*, 55 F.3d 615, 620 (Fed. Cir. 1995). However, where a patentee defines a structurally complete invention in the claim body and uses the preamble only to state a purpose or intended use for the invention, the preamble is not a claim limitation. *Id.*; *Rowe v. Dror*, 112 F.3d 473, 478 (Fed. Cir. 1997).

Obviousness is determined from the context of a person of ordinary skill in the art at the time the invention was made. "[T]he level of skill in the art is a prism or lens through which a judge, jury, or the Board views the prior art and the claimed invention. This reference point prevents these factfinders from using their own insight or, worse yet, hindsight, to gauge obviousness." *Okajima v. Bourdeau*, 261 F.3d 1350, 1355 (Fed. Cir. 2001), (citation omitted). Therefore, the evidence of record must be viewed through the lens of a person of ordinary skill in the art with consideration of common knowledge and common sense. *Graham*, 383 U.S. at 17-18; *DyStar Textilfarben GmbH & Co. Deutschland KG v. C.H. Patrick Co.*, 464 F.3d 1356, 1367 (Fed. Cir. 2006).

Therefore, it is proper to "take account of the inferences and creative steps that a person of ordinary skill in the art would employ." *KSR Int'l Co. v. Teleflex Inc*, 550 U.S. 398, 418 (2007). *See also id.* at 421 ("A person of ordinary skill is also a person of ordinary creativity, not an automaton.").

"In determining whether obviousness is established by combining the teachings of the prior art, the test is what the combined teachings of the references would have suggested to those of ordinary skill in the art." *In re GPAC Inc.*, 57 F.3d 1573, 1581 (Fed. Cir. 1995) (internal quotations omitted). Thus, "[t]he combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results." *KSR*, 550 U.S. at 416. Similarly, "if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill." *KSR*, 550 U.S. at 417.

> "[W]hen a prima facie case is made, the burden shifts to the applicant to come forward with evidence and/or argument supporting patentability." *In re Glaug,* 283 F.3d 1335, 1338 (Fed. Cir. 2002). Rebuttal evidence is "merely a showing of facts supporting the opposite conclusion." *In re Piasecki,* 745 F.2d 1468, 1472 (Fed. Cir. 1984). . . . When a patent applicant puts forth rebuttal evidence, the Board must consider that evidence. *See In re Soni,* 54 F.3d 746, 750 (Fed. Cir. 1995) (stating that "all evidence of nonobviousness must be considered when assessing patentability"); *In re Sernaker,* 702 F.2d 989, 996 (Fed. Cir. 1983) ("If, however, a patent applicant presents evidence relating to these secondary considerations, the board must always consider such evidence in connection with the determination of obviousness.").

In re Sullivan, 498 F.3d at 1351. "When prima facie obviousness is established and evidence is submitted in rebuttal, the decision-maker must start over." *In re Rinehart*, 531 F.2d 1048, 1052 (CCPA 1976); *In re Hedges*, 783 F.2d 1038, 1039 (Fed. Cir. 1986) ("If a prima facie case is made in the first instance, and if the applicant comes forward with

reasonable rebuttal, whether buttressed by experiment, prior art references, or argument, the entire merits of the matter are to be reweighed").

Nevertheless, although secondary considerations must be taken into account, they do not necessarily control the obviousness conclusion. *Newell Companies, Inc. v. Kenney Mfg. Co.*, 864 F.2d 757, 768 (Fed. Cir. 1988). Instead, evidence of secondary considerations are but a part of the "totality of the evidence" that is used to reach the ultimate conclusion of obviousness. *Kansas Jack, Inc. v. Kuhn*, 719 F.2d 1144, 1151 (Fed. Cir. 1983). The weight of secondary considerations may be insufficient to override a determination of obviousness based on primary considerations. *Ryko Mfg. Co. v. Nu-Star, Inc*, 950 F.2d 714, 719 (Fed. Cir. 1991). For example, a long-felt need must not have been satisfied by another before the invention by applicant. *Newell*, 864 F.2d at 768 ("[O]nce another supplied the key element, there was no long-felt need or, indeed, a problem to be solved.").

Additionally,

> In order for a showing of "unexpected results" to be probative evidence of non-obviousness, it falls upon the applicant to at least establish: (1) that there actually is a difference between the results obtained through the claimed invention and those of the prior art . . .; and (2) that the difference actually obtained would not have been expected by one skilled in the art at the time of invention.

In re Freeman, 474 F.2d 1318, 1324 (CCPA 1973) (citations omitted).

Therefore, all of the evidence must be considered under the *Graham* factors before reaching our obviousness determination.

ANALYSIS

The claims stand or fall together (App. Br. 4). Accordingly, we limit our discussion to representative independent claim 40. 37 C.F.R. § 41.37(c)(1)(vii). Claim 40 is drawn to an antivenom pharmaceutical composition for treating a snakebite victim. The claimed composition comprises:

1. Fab fragments which bind specifically to a venom of a snake of the Crotalus genus, and
2. a pharmaceutically acceptable carrier.

Claim 40 also places the following two additional restrictions on the claimed composition:

a. the Fab fragments are essentially free from contaminating Fc as determined by immunoelectrophoresis using anti-Fc antibodies, and
b. the composition neutralizes the lethality of the venom of a snake of the Crotalus genus.

Having defined a structurally complete invention in the body of the claim, we conclude that the recitation of the intended use of the composition "for treating a snakebite victim" as it appears in the preamble of claim 40 does not represent a limitation in this claim. *Rowe v. Dror*, 112 F.3d at 478.

Sullivan teaches an intact horse-derived polyvalent antibody (ACP), or a purified form thereof, that specifically binds to a venom of a snake of the Crotalus genus (FF 8). ACP was the therapeutic modality for treatment of crotalidae envenomation in the United States at the time this invention was made (FF 6). ACP neutralizes the lethality of the venom of a snake of the Crotalus genus (FF 8).

Coulter teaches an anti-Australian brown snake toxin composition comprising Fab fragments that are free of Fc in a pharmaceutically

acceptable carrier (PBS) (FF 13). Coulter teaches that the use of Fab fragments in assays results in a higher sensitivity over the use of intact immunoglobulin molecules (FF 15). Coulter also teaches that Fab fragments retain the ability to neutralize the lethality of snake toxin (FF 16).

Taken together a person of ordinary skill in the art would have recognized that the use of Fab fragments of Sullivan's polyvalent antibody would enhance the sensitivity of Sullivan's antibodies in assays. "[I]f a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill." *KSR*, 550 U.S. at 417. Thus, it would have been prima facie obvious to follow Coulter's methodology to prepare Fab fragments of Sullivan's polyvalent antibody in a pharmaceutically acceptable carrier for use in assays including enzyme immunoassays. Appellants do not dispute and therefore concede that the Fab fragment composition taught by the combination of Sullivan and Coulter would be expected to be essentially free from contaminating Fc as determined by immunoelectrophoresis using anti-Fc antibodies (*see* FF 13).

For the foregoing reasons a person of ordinary skill in this art, at the time the invention was made, who followed the combined teachings of Sullivan and Coulter would have had a reasonable expectation of success in obtaining a composition that (a) is essentially free from contaminating Fc as determined by immunoelectrophoresis using anti-Fc antibodies and (b) neutralizes the venom of a snake of the Crotalus genus.

Accordingly, the combination of Sullivan and Coulter made obvious a composition that comprises:

1. Fab fragments which bind specifically to a venom of a snake of the Crotalus genus, and
2. a pharmaceutically acceptable carrier.

In addition, the combination of Sullivan and Coulter made obvious

a. Fab fragments that are essentially free from contaminating Fc as determined by immunoelectrophoresis using anti-Fc antibodies, and
b. a composition that neutralizes the lethality of the venom of a snake of the Crotalus genus.

Therefore, the combination of Sullivan and Coulter made obvious a composition that neutralizes the lethality of the venom of a snake of the Crotalus genus comprising Fab fragments that specifically bind to a venom of a snake of the Crotalus genus, which are essentially free from contaminating Fc, and a pharmaceutically acceptable carrier.

For their part Appellants do not dispute that the combination of Sullivan and Coulter teaches a composition comprising Fab fragments in a pharmaceutically acceptable carrier for use in assays, such as enzyme immuno-assays (*see e.g.*, FF 15). Instead, Appellants contend that their intended use statement "for treating a snakebite victim" in combination with the claimed requirement that the composition neutralizes the lethality of the venom of a snake of the Crotalus genus *requires* "the Fab fragments [to] exhibit a pharmaceutical activity" (App. Br. 5). While we agree that the Fab component of the claimed composition must neutralize the lethality of the venom of a snake of the Crotalus genus, there is no requirement in claim 40 that this composition be used pharmaceutically, as opposed to its use in performing *in vitro* assays.

Nevertheless, Appellants contend that the "requirement that the antivenom pharmaceutical composition for treating a snakebite victim

comprising Fab fragments neutralizes the lethality of the venom of a snake of the Crotalus genus renders the claims patentable" (*id.*). In support of this contention Appellants direct attention to the First Russell Declaration, the Sullivan Declaration, and the Smith Declaration. Accordingly, faced with Appellants' arguments and Declarations, we reweigh the entire merits of the record before us on appeal. *In re Hedges*, 783 F.2d at 1039. In doing so, we seek to determine, *inter alia*, whether the composition made obvious by the combination of Sullivan and Coulter would have had the same properties as the composition set forth in Appellants' claim 40 – e.g., the ability to treat a snakebite victim with a composition comprising Fab fragments that neutralize the lethality of the venom of a snake of the Crotalus genus (App. Br. 5).

Sullivan teaches an intact horse-derived polyvalent antibody that specifically binds to and neutralizes the lethality of the venom of a snake of the Crotalus genus (FF 8). Sullivan recognizes, however, that immune reactions (such as serum sickness) limit the clinical efficacy of the intact horse derived antibody as an antivenom (FF 10). In this regard, Sullivan teaches that the incidence of serum sickness reactions should be significantly reduced by the removal of extraneous foreign protein (*id.*). Likewise, it was known in the art at the time this invention was made that Fab fragments are less likely to cause undesired immunogenic reactions (FF 11). Coulter teaches a method of producing Fab fragments that are free of Fc and retain their ability to neutralize the toxicity of a snake venom toxin (FF 13 and 16).

Therefore, at the time the invention was made, a person of ordinary skill in this art who followed the combined teachings of Sullivan and Coulter would have had a reasonable expectation of success in obtaining a

composition that (a) is essentially free from contaminating Fc as determined by immunoelectrophoresis using anti-Fc antibodies and (b) neutralizes the venom of a snake of the Crotalus genus. This composition would comprise (1) Fab fragments which bind specifically to a venom of a snake of the Crotalus genus, and (2) a pharmaceutically acceptable carrier. In addition, a person of ordinary skill in this art would have recognized that Fab fragments are less likely to cause undesired immunogenic reactions. Therefore, a person of ordinary skill in this art would have utilized this Fab fragment composition to treat snake bite victims with a reasonable expectation of success given that the polyvalent antibodies taught by Sullivan are based on "the only commercially available antivenom for envenomation by North American snakes of the family Crotalidae" (FF 7) and Coulter teaches that Fab fragments retain their intact parent immunoglobulin's ability to neutralize the lethality of snake venom toxin (FF 16). The test for obviousness is "what the combined teachings of the references would have suggested to those of ordinary skill in the art." *In re GPAC Inc.*, 57 F.3d at 1581. The study discussed in Smith's Declaration and the experimental data in Appellants' Specification confirm the reasonable expectation of success a person of ordinary skill in the art would have had in combining the teachings of Sullivan and Coulter (FF 23 and 24).

Accordingly, we are not persuaded by Appellants' contention that "[t]here was no suggestion in the prior art that Fab fragments could be used to create an antivenom pharmaceutical composition for treating a snakebite victim that would neutralize the lethality of the venom of a snake of the Crotalus genus" (App. Br. 6). For the same reasons we are not persuaded by Appellants' contention that the

> Development of antivenoms comprising antibody fragments [was] halted at the larger $F(ab)_2$ fragments because researchers expected the smaller Fab fragments to be even less effective than $F(ab)_2$ fragments, which appeared to some to be less effective than whole antibody, for several reasons. [First Russell Decl. at ¶ 26; Sullivan Decl. at ¶ 5; Smith Decl. at ¶ 9.]

(App. Br. 8.) On this record, Coulter provides the evidence necessary to establish that Fab fragments are effective in neutralizing the toxicity of snake venom (FF 16). In short, the evidence of record establishes that those of ordinary skill in this art would have reasonably expected that the Fab fragments of Sullivan's polyvalent antibody would not only neutralize the toxicity of snake venom in vivo, but would also provide the additional advantage of reducing serum sickness (*see e.g.*, FF 11). Accordingly, we are not persuaded by Appellants' contention that the use of Fab fragments to neutralize snake venom in vivo would have been unexpected by those of ordinary skill in the art. *See In re Freeman* 474 F.2d at 1324 (to be probative evidence of non-obviousness unexpected results must not have been expected by those of ordinary skill in the art).

Appellants contend that Fab fragments cannot (1) "stearically [sic] hinder[ ] the venom antigen from binding to its active site"; (2) "form cross-linked complexes and precipitate the antigens"; or (3) neutralize venom toxins that continue to be released from the injection site long after the bite because Fab fragments have a half-life of about 17 hours (App. Br. 8-9). We are not persuaded.

There is no evidence on this record to suggest that an immunoglobulin must sterically hinder the binding of the venom to its active site or cross-link and precipitate the venom toxin in order to neutralize the lethality of the toxin. Notwithstanding Appellants' contentions to the contrary, Coulter

teaches that Fab fragments are effective in neutralizing the toxicity of snake venom (FF 16).

We are also not persuaded by Appellants' contention regarding the relationship between the "venom depot effect" (FF 3) and the *in vivo* half-life of circulating antibodies or Fab fragments thereof (FF 18). Initially, it cannot be overstated that the claimed invention is drawn to a composition, not a method of treatment. That said, to the extent that the claimed composition is intended to be used to treat a snake bite, there is nothing in Appellants' claimed invention that would preclude the administration of additional doses of a Fab-based antivenom to maintain the concentration of circulating Fab fragments in the patient at a level that will address the "venom depot effect."

For the foregoing reasons we are not persuaded by Appellants' contentions regarding the long-felt but unsatisfied need in the art to develop antivenoms comprising Fab fragments (App. Br. 9). Appellants' evidence suggests that prior to Coulter, no one in this art took the step toward producing a Fab based antivenom (FF 12). However, prior to Appellants' effective filing date Coulter took that step and taught that Fab fragments are effective in neutralizing the toxicity of snake venom (FF 16). In doing so, Coulter supplied the key element required to satisfy the long-felt need that Appellants contend was recognized in this art (App. Br. 9). *Newell*, 864 F.2d at 768 ("[O]nce another supplied the key element, there was no long-felt need or, indeed, a problem to be solved."). As a result, the composition set forth in Appellants' claim 40 is nothing more than a composition that would have resulted from following the combined teachings of Sullivan and Coulter. Accordingly, we are not persuaded by Appellants' contentions

regarding long-felt need since any such need was satisfied by Coulter prior to Appellants' earliest effective filing date.[2]

Appellants contend that at the time the invention was made "those of ordinary in the skill in the art believe[d] that Fab fragments . . . 'would **increase** the toxicity of the venom' by redistributing and concentrating its toxins. [Sullivan Decl. at ¶ 5(b) (original emphasis), ¶ 13; Russell Decl. at ¶ 33.]" (App. Br. 10 (alteration original)). Appellants contend that "[t]his taxi effect was a reason why those of ordinary skill in the art did not progress beyond the known F(ab)$_2$ fragments to the smaller Fab fragments. [Sullivan Decl. at ¶ 7]" (*id.* (alteration original)). To support this contention Appellants rely on the post-filing date Faulstich reference. We are not persuaded.

Faulstich teaches that both intact antibody against the mushroom toxin (α-amatoxin) and Fab fragments thereof are incapable of neutralizing the toxicity of the toxin (FF 20). Instead, Faulstich found that the Fab fragments increased the toxicity of the α-amatoxin (FF 21). In this regard, the post-filing date Faulstich reference reports that "[t]o our knowledge this is the first reported case where immunoglobulins or their fragments enhance rather than decrease the activity of a toxin" (*id.*). While the post-filing date Balthasar reference cites Faulstich, it adds nothing more than a comment that "[t]he risk of redistributing systemic toxicity, rather than minimizing

[2] We recognize the argument Appellants made in their Brief to our appellate reviewing court regarding commercial success. 2006 WL 3243570 50-51; 2006 WL 4385792 17-18. We note, however, Appellants provided no evidence or argument relating to commercial success in any of their briefings to this Board.

systemic toxicity, should be appreciated as a potential outcome of the proposed approach" (FF 21; App. Br. 12).

Accordingly, both Faulstich and Balthasar fail to support Appellants' contention that, at the time their invention was made, those of ordinary skill in this art were concerned that Fab fragments of antibodies, *effective in neutralizing the toxicity of toxins*, would increase the toxicity of the toxin due to what Appellants refer to as a "taxi-effect."

In contrast to Faulstich, the combination of references relied upon on this record teach antibodies and Fab fragments that *are effective in neutralizing the toxicity of a toxin*. Specifically, Coulter teaches that Fab fragments are effective in neutralizing the toxicity of snake venom (FF 16). As Appellants admit, the post-filing date Sorkine reference conducted a study similar to Coulter's and obtained similar results (App. Br. 13).

In sum, we have not been directed to sufficient evidence on this record to support Appellants' intimation that Fab fragments derived from antibodies that were *capable of neutralizing the toxicity of a toxin*, as taught by the combination of Sullivan and Coulter, would increase - rather than neutralize - the toxicity of a toxin.

Nevertheless, Appellants contend that Coulter did not teach the treatment of "envenomation with their Fab fragments," rather Coulter pre-mixed the Fab fragments with the toxin and then injected the complex into mice to determine if the Fab fragments were capable of protecting the mouse from the effects of the toxin (App. Br. 13). Appellants contend that "[s]ince the Fab-textilotoxin mixture was first mixed *in vitro* and then injected intravenously, the Fab did not have the opportunity to redistribute and concentrate the textilotoxin in high blood flow parts" (*id.*). Further,

Appellants contend that Sorkine's post-filing date teachings establish "that one would not have expected Coulter et al.'s in vitro neutralization results to predict the effectiveness of antivenoms comprising Fab fragments in vivo" (App. Br. 14 (emphasis removed)). We are not persuaded.

Sorkine teaches that if antibody fragments are not mixed with venom prior to injection then a larger concentration of antibody fragments is necessary to neutralize the venom (FF 22). This is a dosage issue well within the purview of a person of ordinary skill in the art at the time the invention was made. Further, Sorkine supports rather than refutes the conventional knowledge in the art at the time the invention was made, by teaching that due to their faster diffusion and a greater volume of distribution Fab fragments would be more effective at neutralizing venom than intact antibody and $F(ab)_2$ fragments (*Cf.* FF 22 and FF 17).

Despite Appellants' contention to the contrary, Coulter utilized what appears to have been an art accepted procedure for predicting efficacy of antivenom at the time of the claimed invention. In this regard, we recognize that Appellants utilize a similar procedure to determine efficacy of antivenom as does Sullivan (FF 19). Further, according to Faulstich the toxicity of a toxin administered i.p. followed by the i.v. administration of a monoclonal antibody "is very similar to the toxicity caused by i.v. administration of the amanitin-immunoglobulin complex" (*id.*). Therefore, despite Appellants' contention to the contrary, Faulstich confirms that methodology utilizing a pre-formed complex of a toxin and an antibody or fragment thereof is predictive of the separate administration of the two compounds (*id.*).

In addition, Appellants contend that since Coulter's Fab fragments were directed to a single toxin in the venom of the Australian brown snake "the Examiner is incorrect in attempting to extrapolate Coulter['s] . . . results with Fab fragments to a single snake venom toxin to the results that would have been expected with Fab fragments to an entire snake venom" (App. Br. 14). Accordingly, Appellants contend that "[s]ince one of ordinary skill in the art would not have expected Coulter['s] . . . results with Fab fragments to a single venom toxin to predict what would occur with an antivenom comprising Fab fragments to an entire venom, any rejection relying upon the Coulter *et al.* reference must fail" (App. Br. 14-15). We are not persuaded.

Notwithstanding Appellants' contention to the contrary, the prior art suggests the use of Coulter's methodology to prepare Fab fragments of Sullivan's polyvalent antibody. Thus, the result would not be a Fab fragment directed toward a single toxin, but instead would be antivenin polyvalent Fab fragments directed against a plurality of toxins in the venom of a snake in the Crotalus genus (FF8).

We recognize the Sullivan Declaration's discussion of equine-derived IgG(T) antibodies such as those taught by Sullivan (Sullivan Declaration 4: ¶ 6). The Sullivan Declaration states that "[t]he early success of equine-derived antivenin containing IgG(T) antibody was due to the nature of the IgG(T) antibody, which has an extra disulfide bond . . .[, which] allows IgG(T) to bind with enhancement to repeating protein antigens" (*id.*). The Sullivan Declaration states that while IgG(T) and its corresponding $F(ab)_2$ fragment would both contain this "extra disulfide bond," a "F(ab) would <u>not</u>, thus diminishing clinical efficacy" (Sullivan Declaration 4-5: ¶ 6). We are not persuaded.

Coulter teaches rabbit-derived Fab fragments, which do not contain the "extra disulfide bond" found in IgG(T) antibodies, that were effective in neutralizing the toxicity of snake venom thereby demonstrating that the "extra disulfide bond" is unnecessary to neutralize the toxicity of snake venom (FF 16).

CONCLUSIONS OF LAW

For the foregoing reasons, the conclusion of obviousness over the combination of Sullivan and Coulter stands when reconsidered in view Appellants' arguments and Declaratory evidence on this record.

The rejection of claim 40 under 35 U.S.C. § 103 as being unpatentable over the combination of Sullivan and Coulter is affirmed. Claims 41, 42, and 50 fall together with claim 40.

The rejection of claims 40-42 and 50 under 35 U.S.C. § 103 as being unpatentable over the combination of Sullivan, Coulter, Smith, and Stedman's is affirmed for the reasons discussed above. Smith and Stedman's are cumulative.

TIME PERIOD FOR RESPONSE

No time period for taking any subsequent action in connection with this appeal may be extended under 37 C.F.R. § 1.136(a).

AFFIRMED

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Simplified Preparation of Rabbit Fab Fragments

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Papain attached to solid-phase CH-Sepharose 4B was used to digest rabbit IgG. Protein A–Sepharose CL-4B was used to remove undigested IgG and Fc fragments. Pure Fab fragments free of IgG, Fc fragments and papain were readily obtained by this procedure with a yield of about 75%. Polyacrylamide gel electrophoresis of the Fab in the presence of sodium dodecyl sulphate gave a single band under both reducing and non-reducing conditions. The molecular weight of the Fab determined by sedimentation equilibrium was 49,200. Unlike the IgG, the Fab obtained did not form precipitin lines when used in immunoelectrophoresis.

Key words: *rabbit Fab — solid-phase papain — protein A*

Introduction

Fab fragments of IgG have been used in enzyme immunoassay (EIA) instead of IgG (Kato et al., 1976). EIAs of higher sensitivity have been claimed when Fab enzyme is used instead of IgG enzyme. The original method of Porter (1959) is a rather tedious procedure for preparing rabbit Fab fragments, although it is still widely used. Our requirement for such fragments arose from an investigation of the binding site(s) of snake neurotoxin at the neuromuscular junction. For this purpose a Fab enzyme conjugate was needed. We have used solid-phase techniques to simplify the preparation of rabbit Fab fragments.

Materials and Methods

Rabbit IgG

Immune and normal rabbit IgG were prepared by affinity chromatography of crude serum on protien A–Sepharose CL-4B (Pharmacia). The method used was that of Goding (1976) as modified by Coulter et al. (1980). The immune serum had been raised against a neurotoxin (textilotoxin) isolated from the venom of the Australian brown snake, *Pseudonaja textilis*.

Solid-phase papain

Five milligrams of papain (E.C. no. 3.4.22.2) type III, twice recrystallised, obtained from Sigma were reacted with 0.5 g of activated CH-Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

Ninety-one per cent of the papain was covalently bound to the solid phase. Unbound papain was estimated by the Folin–Lowry procedure (Lowry et al., 1951).

The solid phase was stored as a 10% v/v suspension at 4°C in 0.05 M phosphate-buffered saline, pH 7.4 (PBS) containing 0.1% sodium azide.

Preparation of Fab

The procedure used was that described by Hudson and Hay (1976) except for the use of solid-phase papain and protein A to obtain Fab fragments.

The following reagents were added to 10 mg of rabbit IgG in 1 ml of PBS: 0.2 ml of 16 mg/ml cysteine hydrochloride, 0.2 ml of 8 mg/ml EDTA, sodium salt, 1 ml of solid-phase papain (equivalent to 1 mg), washed with 0.15 M phosphate, pH 7.0, to remove sodium azide.

The mixture was incubated with gentle stirring at 37°C for 4 h. The solid phase was sedimented by gentle centrifugation (1200 × *g* for 5 min) and the supernatant washed through a 4 ml protein A–Sepharose CL-4B column with 10 ml of PBS (pool A). Undigested IgG and Fc fragments were eluted from the column by successive washings with: 10 ml of PBS containing 0.1% Tween 20: this was discarded. Ten millilitres of PBS, discarded. Ten millilitres of 0.1 M glycine/HCl containing 1 M NaCl, pH 3. This was collected into 10 ml of PBS and adjusted to pH 7.4 with 1 M NaOH (pool B).

The column was finally stored in PBS containing 0.1% sodium azide.

Pools A and B were concentrated separately to about 5 ml by ultrafiltration over a PM-10 membrane in a model 52 cell (Amicon).

IgG and Fab concentrations were determined with values of $E_{279\,nm}^{1\%} = 14.0$ and 14.8 respectively (Mandy and Nisonoff, 1963).

Immunoelectrophoresis (IEP)

IEP was performed by the method of Scheidegger (1955). The antiserum used was rabbit anti-textilotoxin.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Weber and Osborn (1969) and Laemmli (1970). SDS was obtained from British Drug Houses as were acrylamide and methylenebisacrylamide. TEMED and 2-mercaptoethanol (2-ME) were from Eastman Organic Chem. Protein molecular weight standards were obtained from Pharmacia.

Analytical ultracentrifugation

The molecular weight determination was done by meniscus depletion in a Beckman model E analytical ultracentrifuge. The method of Yphantis (1964) was used. A $\bar{v}$ value of 0.73 cc/g was assumed for rabbit Fab.

Enzyme immunoassay (EIA)

EIA was used to determine the specific antibody titre after preparation of Fab from IgG. The EIA was as described previously (Coulter et al., 1981) for direct assay of snake venom. The wells of polystyrene microhaemagglutination plates (u-wells, Cooke Lab. Prod.) were coated with textilotoxin. The following procedure was used.

Plates were floated in a water bath at 37°C for 2 h after adding 0.1 ml of 1 μg/ml textilotoxin in 0.05 M sodium carbonate buffer, pH 9.6, to the wells. The plates were washed and incubations performed as previously described (Coulter et al., 1981). The relative ability of rabbit IgG anti-textilotoxin and Fab anti-textilotoxin to block the uptake of rabbit IgG anti-textilotoxin–horse radish peroxidase (HRPO) conjugate by the polystyrene bound textilotoxin was determined in the following way. Serial dilutions of IgG and Fab were incubated in wells coated with textilotoxin for 30 min at 37°C. After washing and subsequent reaction with conjugate the degree to which IgG and Fab had blocked conjugate uptake was determined by intensity of colour development on addition of substrate to the wells.

Mouse protection assays

IgG and Fab anti-textilotoxin were tested for their ability to neutralise the lethal effects of textilotoxin in mice. Eighteen to 21 g Swiss mice (CSL strain) were used in the assay. Four mice were used at each dose level and all injections were given intravenously. Four LD_{50} of textilotoxin were incubated with serial dilutions of immune IgG and Fab. All doses were given in a volume of 0.2 ml. A 0.1% solution of bovine serum albumin in 0.85% NaCl was used as diluent; this reduces non-specific adsorption of textilotoxin (Broad et al., 1979).

IgG and Fab prepared from normal rabbit IgG were used as controls.

Ion exchange chromatography of Fab prepared by the solid-phase papain/protein A procedure

Fab prepared by the solid-phase papain/protein A procedure was subjected to ion exchange chromatography. The method of Porter (1959) as modified by Hudson and Hay (1976) was used.

Results and Discussion

Fab fragments prepared by the solid-phase papain/protein A procedure gave a single diffuse band on SDS-PAGE run under non-reducing conditions. A single band of M_r 27,000 was obtained on SDS-PAGE run under reducing conditions (Fig. 1). Rabbit Fab and Fc fragments have M_rs of about 50,000 (Marler et al., 1964). No IgG was detectable by SDS-PAGE in the Fab preparation.

A M_r of 49,200 was obtained by sedimentation equilibrium. The M_r was constant through most of the solution column, with a slight increase towards the base of the cell.

Dialysis of the Fab preparation against a nominal 10,000 M_r cut-off membrane ensures removal of low M_r products. Apart from the Fab band, no other high or low

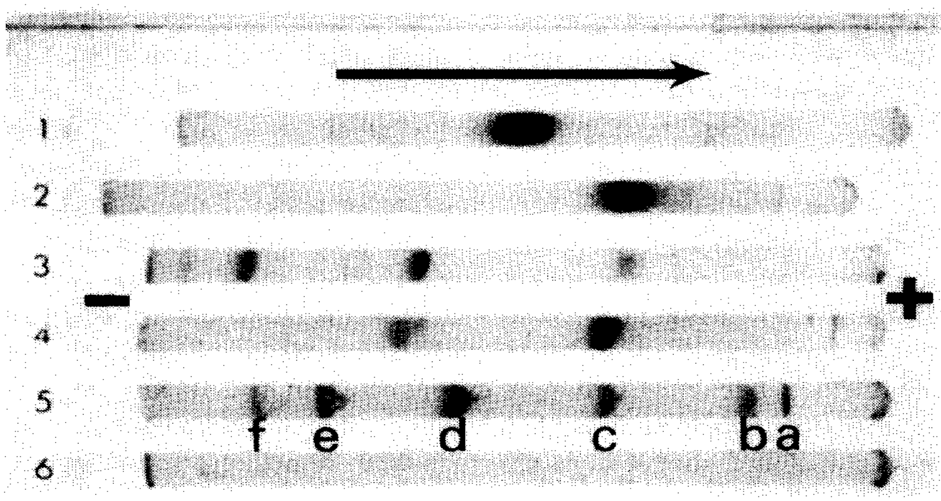

Fig. 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Laemmli, 1970) of rabbit IgG and its fragments resulting from solid-phase papain digestion. Essentially the same results were obtained with the Weber and Osborn (1969) gel system (not shown). Gel 1: Non-protein A bound material after solid-phase papain digestion, i.e., Fab run under non-reducing conditions. Gel 2: Fab prepared by the solid-phase papain procedure. Sample reacted with 2-ME. Gel 3: protein A bound components after solid-phase papain digestion. These were eluted at low pH. Gel run under non-reducing conditions. Gel 4: sample as for gel 3, treated with 2-ME. Gel 5: protein M_r standards: a, α-lactalbumin, 14,400; b, trypsin inhibitor, 20,100; c, carbonic anhydrase, 30,000; d, ovalbumin, 43,000; e, albumin, 67,000; f, phosphorylase b, 94,000. Gel 6: blank gel.

M_r products were visible by SDS-PAGE.

When the Fab preparation was fractionated by ion exchange, 94% of the starting material was recovered. Sixty-five per cent of the starting material was eluted with the equilibrating buffer (0.01 M sodium acetate, pH 5.5) and 29% was eluted before a sodium acetate concentration of 0.2 M was reached. No other material was eluted, even when 1 M sodium acetate, pH 5.5, was used.

When the anti-textilotoxin titre of the Fab preparation was compared with that of IgG by EIA, the Fab concentration which yielded 50% of full colour in the assay was 4.5 μg/ml. The IgG concentration which gave the same colour was 6.2 μg/ml. In terms of anti-textilotoxin activity in the EIA, when their relative M_rs are considered, the Fab and IgG were practically equivalent. The final yield of Fab was always about 75% of that theoretically possible, i.e., 5 mg Fab from 10 mg of IgG.

The neutralisation tests performed in mice showed that on a weight basis the IgG and Fab anti-textilotoxin preparations were equivalent in neutralising ability, i.e., a loss of approximately 30% of specific antibody activity.

When equivalent concentrations of Fab and IgG anti-textilotoxin were tested by IEP against textilotoxin, precipitin lines were obtained with IgG but not with the Fab preparation.

These results demonstrate that Fab fragments can be obtained from rabbit IgG with losses of 20–30% of initial IgG antibody activity. The steps involved in the procedure are relatively simple. The solid-phase papain is not difficult to prepare;

the reaction with IgG is straightforward as in the protein A purification of the Fab. The procedure represents a marked improvement in terms of simplicity and time saving over the ion exchange procedure for the preparation of rabbit Fab fragments.

Note Added in Proof

Since completing this paper an important article came to our attention describing the same experimental procedure:

Goding, J.W., 1978, J. Immunol. Methods 20, 241.

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ISOLATION AND PURIFICATION OF ANTIBODIES TO RATTLESNAKE VENOM BY AFFINITY CHROMATOGRAPHY

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The present therapeutic modality for treatment of Crotalidae envenomation in the United States involves the intravenous administration of equine source Antivenin (Crotalidae) Polyvalent (ACP). Two basic problems are associated with the use of ACP. Firstly, it is difficult to determine the amount of ACP required to mitigate the venom effects in a patient and thus large amounts of horse protein may need to be administered. Secondly, both immediate and delayed sensitivity reactions to horse serum and the treatment of patients known to be sensitive to horse proteins present a problem.

Some attempt has been made to circumvent the use of horse serum in sensitive patients by preparing antivenins in other animals (1). Also, some critical care units have allowed for the treatment of horse serum sensitive individuals with ACP under intensive monitoring and support (2). However, the need for a more highly purified immunopharmacologic agent devoid of sensitivity reactions has become evident. The present report deals with investigations into the isolation and purification of antibodies from ACP by affinity chromatographic techniques and pharmacologic studies on these purified antibodies. Finally, attention was given to the problem of anaphylactic reactions to the purified antivenin.

METHODS: Affinity chromatography using acrylamide gel was employed to isolate and purify antibodies to four rattlesnake venoms: *Crotalus atrox, C. adamanteus, C. scutulatus scutulatus* and *C. viridis helleri*. An individual column was prepared with a separate venom as affinant. Each column was prepared by dissolving 100 mg of lyophilized venom in 10 ml of acrylamide monomer (16% acrylamide, 4% N,N-methylene-bisacrylamide) in phosphate buffered saline, pH 7.4, in a small beaker. Polymerization of the venom-acrylamide mixture was achieved by addition of 500 µl of 0.4% ammonium persulfate in water and 50 µl of TEMED (N,N,N-N-tetramethylethylenediamine). The mixture was well mixed and water was layered over the surface to exclude oxygen. The venom-acrylamide mixture gelled in 10 min and then was triturated by forcing broken pieces through a plastic syringe fitted with successively smaller needles from 18 gauge to 21 gauge. Phosphate buffered saline (PBS) was added to the syringes to facilitate gel pulverization. This process was repeated until the gel easily passed through the 21 gauge needle. At this point, the venom-acrylamide gel was reduced to a gritty consistency. The pulverized gel was defined 5-6 times with PBS and packed by gravity into columns 1 cm x 20 cm. The venom-acrylamide columns were washed with alternating cycles of PBS and 0.1 M glycine, pH 2.5 (0.1 M glycine, 0.154 M NaCl, pH adjusted with HCl) until a steady baseline was obtained by monitoring the

effluent at 280 nM with a spectrophotometer. The column was returned to pH 7.4 with PBS and was then ready for use.

Commercial Antivenin (Crotalidae) Polyvalent diluted 10 ml was applied to each column. A column flow rate of 0.5 ml/min was controlled with a pump and protein detection of the effluent was monitored with a spectrophotometer at 280 nM. The initial peak eluted with PBS consisted of extraneous protein. After the effluent returned to baseline, the solvent was changed to 0.1 M glycine, pH 2.5 and a second peak was eluted which consisted of purified antibody (SRA). This effluent was collected separately. The column was then restored to pH 7.4 with PBS for reuse. The pH of the antibody effluent was adjusted to 7.4 with TRIS buffer. The isolated, purified antibody effluent was dialyzed against distilled water overnight at 10°C, lyophilized and stored at -20°C. Purified antivenin was isolated to each of the four venoms by acrylamide affinity chromatography as described.

Microbiuret protein assays were performed for each antivenin following dialysis and lyophilization and also for the lyophilized product (Table 1).

The specificity of the purified, isolated antibodies for *Crotalus* venom compared to the original antivenin was determined by immunoelectrophoresis of each specific venom developed with both the specific isolated SRA and the material (Fig. 1). The purity of the isolated SRA was demonstrated by immunoelectrophoresis of both isolated antibodies and the antivenin developed with anti-horse serum (Fig. 2).

The intravenous LD_{50} for each venom studied was determined and multiples of this were mixed with two to ten times the same amounts of purified SRA, ACP or bovine serum albumin (BSA). Individual solutions of venom purified antibody, venom ACP and venom BSA were allowed to stand for 30 min before use in lethality determinations. Swiss-Webster mice, weighing 20-26 g, were administered the individual solutions by tail vein in volumes less than 100 µl. Results were interpreted at the end of 24 h. Each purified antibody and the ACP was tested against each separate venom, using BSA as a control. Tables 2 and 3 list the results of two of those studies.

The ability of the purified antibody to prevent fibrinogen clotting induced by *C. adamanteus* venom was studied by the method of Bajwa & Markland (3). The purified antibody was compared to ACP and to a control (Table 4).

Human embryonic lung cell cultures were incubated for 30 min with saline or human serum and with varying doses of *C. v.h.* venom. The tissue damage was recorded on a scale of 0 (no damage) to 4+ (complete cellular destruction and disruption). A second group of cultures was then incubated with varying amounts of the venoms of *C. ad.*, *C. vh.* and *C. s.s.* according to the method of Wingert (4). The microgram amount of the purified antibodies of *C. v.h.*, *C. ad.* and *C. s.s.* to prevent cytotoxicity was determined for 20 µg of *C. v.h.* venom, 50 µg of *C. ad.* venom and 60 µg of *C. s.s.* venom, respectively. In addition, the microgram amount of ACP needed to prevent cytotoxicity from *C. v.h.* venom (20 µg) was determined (Table 5).

The ability of the purified SRA to prevent or reduce subcutaneous hemorrhage was compared to the ACP, with BSA as a control. Various amounts of venom from one tenth the intravenous LD_{50} were mixed with purified SRA, ACP and BSA in amounts two to ten times that of the venom. These solutions were allowed to stand 30 min and then injected subcutaneously over the lateral dorsal area in mice. Two hundred mice were employed in this study. Results were interpreted at 24 h

by noting the size of the hemorrhagic areas (Fig. 3).

The hypersensitivity studies were conducted in two separate experiments. The first preliminary investigation involved sensitizing guinea pigs to ACP and challenging with purified SRA, ACP or BSA. The second investigation involved assaying the release of histamine from leukocytes of an individual sensitive to horse serum by the method of Lichtenstein & Osler (5).

RESULTS: The specificity of the purified SRA was demonstrated by immunoelectrophoresis of individual venoms with the separate purified antivenins and the ACP. This revealed multiple precipitin bands with the SRA and very few precipitin bands with the ACP (Fig. 1). This was to be expected because of the concentration of specific antibodies to the venom antigens. Fig. 2 demonstrates the relative purity of the isolated SRA as compared to ACP, when both are immunoelectrophoresed and developed with rabbit anti-horse serum. The purified SRA shows a single dense precipitin band consistent with the immunoglobulin portion of horse serum, whereas the ACP demonstrates multiple bands consistent with albumin and other horse proteins. It is evident that the extraneous protein, including albumin, has been eliminated from the SRA product.

Table 2 shows the results of injecting two times the LD_{50} of *C. ad.* venom mixed with four times the weight of one LD_{50} of each species specific purified SRA, ACP and BSA. Table 3 shows the results of injection of two times the LD_{50} of *C. v.h.* venom with five times the weight of one LD_{50} of each species specific purified SRA, ACP and BSA. In each case, the species specific antivenin provided almost complete protection against lethality to its specific venom. There was some degree of cross protection provided by the *C. ad.* purified SRA when studied against the *C. v.h.* venom (Table 3).

Fibrinogen clotting results (Table 4) demonstrated protection against clotting by both *C. v.h.* and *C. ad.* antibody, whereas ACP demonstrated no protection at a ratio of 1:2.4. These are compared to a control clot occurring at 34 sec. At a ratio of 1:4.8 venom, ACP provided only weak protection, with a clot occurring at 51 sec.

Cytotoxicity results revealed good protection against cellular destruction by purified SRA (Table 5). ACP also protected against cytotoxicty from *C. ad.* venom but required almost three times the SRA dose.

The ability of ACP and the SRA to mitigate the hemorrhagic effects of the individual venoms was evident in most every animal studied. The greatest protection was shown in the cases of *C. ad.* and *C. v.h.* with the species specific antivenins. Cross protection tests were slightly less protective in the cases of *C. at.* and *C. s.s.* In each test animal, the SRA was more effective than the ACP, when specific venom-antivenin combinations were studied, and in many cases species specific cross protection was evident. BSA afforded no protection. Fig. 3 is representative of the hemorrhagic results, showing the protective effects of SRA compared to ACP and BSA.

Preliminary guinea pig sensitization studies (Table 6) demonstrated that hypersensitivity reactions occurred in the guinea pigs challenged with ACP and BSA but no reactions occurred in the animal challenged with SRA. These tests are being repeated in larger animal groups to confirm this preliminary data. *In vitro* leukocyte histamine release to antigenic stimulus demonstrated the potential for acute hypersensitivity reaction. Table 7 summarizes *in vitro* data of a patient who

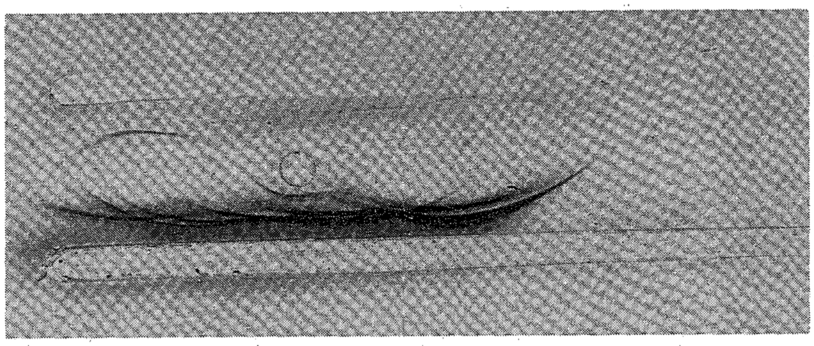

FIGURE 1: Immunoelectrophoresis of ACP (top light bands) and SRA, *C. adamanteus*, (bottom dark bands) developed with *C. adamanteus* venom.

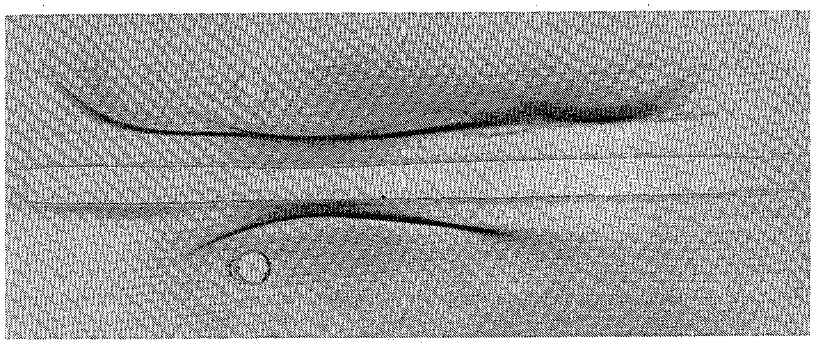

FIGURE 2: Immunoelectrophoresis of ACP (top dark bands) and SRA, *C. atrox*, (bottom single band) developed with rabbit anti-horse serum.

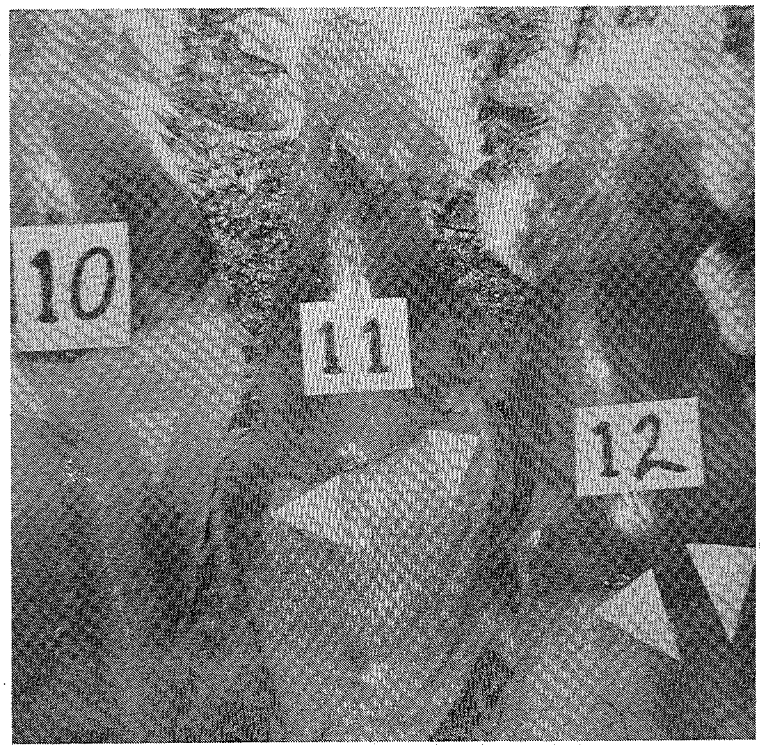

FIGURE 3: Results of hemorrhagic study: Number 10 shows ACP on left with BSA on right (arrow points to large hemorrhagic areas with both); Number 11 shows BSA on left with SRA on right; note lack of hemorrhage with SRA; Number 12 shows both ACP and BSA showing large hemorrhages.

suffered anaphylaxis to a horse serum skin test after being envenomated by a rattlesnake. These data indicate that this patient is very sensitive to foreign antigens with a spontaneous histamine release of 55% in standard human plasma. Base line histamine release in this patient was 8%, when his leukocytes were incubated in his own plasma. SRA antigenic stimulation caused a 12% histamine release from leukocytes, whereas ACP caused a 50% release and horse serum caused a 55% release.

TABLE 1: Protein assay

Antivenin 200 μg/ml	% Purity
C. atrox	77
C. adamanteus	74.4
C. viridis helleri	89.5
C. scutulatus scutulatus	77.8
ACP	61.8

TABLE 2: Lethality determinations of *Crotalus adamanteus* venom and antisera

Product	No. mice	No. dead	No. alive
ACP	10	8	2
SRA, *C. ad.*	10	0	10
SRA, *C. at.*	10	8	2
SRA, *C. v.h.*	10	7	3
SRA, *C. s.s.*	10	6	4
BSA	10	10	0

TABLE 3: Lethality determinations of *Crotalus viridis helleri* venom and antisera

Product	No. mice	No. dead	No. alive
ACP	10	9	1
SRA, *C. ad.*	10	4	6
SRA, *C. at.*	10	8	2
SRA, *C. v.h.*	10	1	9
SRA, *C. s.s.*	10	8	2
BSA	10	10	0

DISCUSSION: Preliminary investigation of the neutralization capacities of SRA and ACP involved lethality protection, fibrinogen clot prevention, prevention of cytotoxicity, protection against hemorrhage and sensitivity studies. These investigations, although not complete, indicate that the purified antivenin (SRA) isolated by affinity chromatography is more efficacious than the commercially available antivenin. The data on sensitivity reactions are not complete and detailed immunological studies are being conducted; however, our preliminary data indicated that the purified SRA would be less likely to produce acute anaphylaxis in individuals sensitive to horse serum than would ACP. Also, due to the removal of

TABLE 4: Fibrinogen clotting results

C. ad. venom (5 mg/ml)	Antivenin	Ratio	Clotting time
1) 0.1 ml	Control buffer	--	34 sec
2) 0.1 ml	0.2 ml *C. ad.* AB (6 mg/ml)	1:2.4	No clot
3) 0.1 ml	0.2 ml *C. v.h.* AB (5 mg/ml)	1:3	No clot
4) 0.1 ml	0.2 ml ACP (6 mg/ml)	1:2.4	33 sec
5) 0.1 ml	0.4 ml ACP (6 mg/ml)	1:4.8	51 sec

TABLE 5: Cytotoxicity studies

Tissue culture: Embryonic lung

Venom	Antivenin	30 min post-incubation results
C. v.h. 20 μg	--	4+ Cytotoxicity
C. v.h. 20 μg	*C. v.h.* 89.5 μg	No Cytotoxicity
C. v.h. 20 μg	ACP 247.2 μg	No Cytotoxicity
C. ad. 50 μg	--	4+ Cytotoxicity
C. ad. 50 μg	*C. ad.* 74.4 μg	2+ Cytotoxicity
C. ad. 50 μg	*C. ad.* 93.0 μg	No Cytotoxicity
C. s.s. 60 μg	--	4+ Cytotoxicity
C. s.s. 60 μg	*C. s.s.* 140.0 μg	No Cytotoxicity

extraneous foreign protein, the incidence of serum sickness reactions should be significantly reduced.

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TABLE 6: Guinea pig sensitization

Guinea pig	Challenging agent/dose		Result
#1	ACP	1 mg	Sneezing, scratching, difficulty breathing; survived
#2	ACP	1 mg	Sneezing, scratching; survived
#3	SRA (*C. v.h.*)	1 mg	No reaction
#4	BSA	1 mg	Death in 5 min

TABLE 7: Isotopic histamine release assay from basophils

Patient's cells	% Release
PT cells - *St'd serum* + 25 µl buffer*	55
PT cells - *PT serum* + 25 µl buffer	8
PT cells - *PT serum* + 25 µl 1:10 horse serum	55
PT cells - *PT serum* + 25 µl ACP (1 mg/ml)	50
PT cells - *PT serum* + 25 µl purified *C. ad.* AB (1 mg/ml)	12

*TRIS buffer + Ca^{++} + Mg^{++}

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Envenomings and their Treatments

Edited by C. Bon and M. Goyffon

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Eds C. Bon and M. Goyffon
Envenomings and their Treatments

Use of antibodies as antivenoms: A primitive solution for a complex problem?

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Résumé

L'envenimation ophidienne est un processus complexe qui met en jeu de multiples variables, tant dans le venin que chez la victime. Les composants du venin varient, en dimension, du cation métallique à la grosse protéine multimérique. Ces composants peuvent provoquer des lésions locales ou affecter les systèmes nerveux, sanguin, cardiovasculaire et immunitaire. Un antivenin doit être capable de neutraliser les composants actifs du venin. De plus, il doit être bien toléré, facile d'utilisation et bon marché. Par le passé, les antivenins étaient des médicaments relativement primitifs. Bien qu'ils aient été considérés comme efficaces, leur utilisation était handicapée par des réactions allergiques aiguës ou retardées, et souvent limitée par une dissolution lente et une courte durée de conservation. Les antivenins récents gomment ces imperfections grâce à l'élimination de composants indésirables du sérum antivenimeux et à la sélection des anticorps spécifiques. Les premières constatations montrent que les nouveaux antivenins peuvent effectivement bloquer les effets du venin tout en évitant les effets secondaires des premières fabrications.

Summary

Snake venom poisoning is a complex process which involves multiple variables in both the venom and the victim. Snake venoms contain many components, ranging in size from metals to large multi-unit proteins. These components may cause local injury as well as affect the neurological, haematological, cardiovascular and immune organ systems. An antivenom must be capable of neutralizing the injurious components of the venom. In addition, safety, reasonable cost and ease of use are important. In the past, antivenoms were relatively primitive drugs; although generally considered effective, their use was plagued by acute and delayed allergic reactions, and they were often difficult to handle because of slow dissolution times and short shelf lives. Newer antivenoms overcome these shortcomings by eliminating the unwanted components of animal sera and incorporating only those antibodies specific to the target venom. Preliminary evidence in several types of envenoming indicates that the newer antivenoms can effectively halt the effects of venoms without the untoward effects associated with earlier products.

Introduction

Antibody drugs derived from animal sera may be the most misunderstood and undervalued pharmaceutical products. Although the efficacy and mechanism of action of animal sera have been known for nearly 100 years, antibodies have

never assumed a major role in the treatment of disease. Today, the term 'antibody' often calls to mind the financial losses caused by the dismal performance of monoclonal antibodies as therapeutic agents. While monoclonal antibodies are extraordinary tools that have revolutionized molecular and cellular biology, they are distinct from polyclonal antibodies. The defining characteristic of a monoclonal antibody is specificity. By manipulating clonal selection, a line of antibodies that recognizes only one antigenic determinant can be produced. Monoclonal antibodies can thus precisely identify small differences between molecules. If a monoclonal antibody is the 'A' in an alphabet soup, however, polyclonal antibodies are a soup containing all the letters of the alphabet. Polyclonal antibodies are the product of the typical immune response developed by animals. They are formed to all antigenic determinants recognized by the host's immune system. Thus, the immune response is a soup of antibodies from 'A' to 'Z'. This concept is important because precision is not necessarily the most desirable characteristic of a therapeutic antibody. The term 'therapeutic antibody' implies that the antibody prevents or reverses the action of an antigen like an antivenom, preventing the effects of envenoming. An antibody may, however, bind an antigen in the wrong location and thereby fail to neutralize its action. Thus, the specificity of monoclonal antibodies is a two-edged sword: If it binds to the wrong antigenic determinant, it will be ineffective.

One disease process that has been successfully treated with polyclonal antibody products is snake venom poisoning. Since the pioneering work of Sewall (1887), Calmette (1894) and Phisalix and Bertrand (1894) over 100 years ago, the polyclonal antibodies in animal sera have been a known as a simple, sure method for the treatment of nearly any type of envenoming.

Snake venom poisoning

Snake venom poisoning may seem simple, but it is remarkably complex. Snake venoms are a mixture of numerous components ranging from small metal complexes, through peptides to complex proteins with relative molecular masses in excess of 100 (Bieber, 1979). Venoms contain many types of compounds, including metals and other inorganic compounds, some lipids and carbohydrates, riboflavin, nucleosides and nucleotides, amino acids and peptides, amines and multiple types of enzymes (Bieber, 1979; Iwanaga & Suzuki, 1979). The components of snake venom may produce local effects only, systemic effects only, or both. The number of venom components varies a great deal, but all snake venoms contain several toxic components, which led Nicole and Bouquet (1925) to describe snake venoms as a 'mosaic of antigens'.

It has become apparent recently that the composition of venom mixtures changes over time. For reasons that are unclear, the composition of rattlesnake venom varies from snake to snake within the same species and from season to season, even within the same snake, particularly as the snake matures (Bonilla *et*

al., 1973). For example, the LD_{50} values of seven individual specimens of *Crotalus horridus horridus* varied fivefold in specimens collected from the same locale and tested simultaneously (Minton, 1953). Similar results have been obtained with other species (Glenn & Straight, 1978). This variability complicates the development of an antivenom product, which must neutralize the toxic effects of a venom at any time of the year and from all snakes in a species.

Most venoms have some solid precipitate in addition to the components in solution. A venom can therefore be considered as a heterogeneous solution and suspension of venom fractions in an aqueous base. Since the precipitant is difficult to work with, it is usually removed by centrifugation before injection into an animal. It is unclear whether this portion of the venom is injected during the act of envenoming, but it is clear that with current techniques this fraction is not used to immunize an animal.

After being injected, venom is absorbed. This means that it must pass through membranes to gain access to the vascular system, by which it can be distributed throughout the body. It is likely that certain components of the venom are absorbed more quickly than others, depending on their physicochemical characteristics, and it is reasonable to assume that the absorption kinetics are different for each component. The smaller, water-soluble components will be absorbed first, while large, insoluble components will be absorbed last. It not difficult to envision that the absorption of some components could span many hours.

A further complication of antivenom treatment is that the site of venom deposition during a bite also varies. Venom may be deposited in the subcutaneous tissues or muscle; in rare cases, it may be injected directly into a blood vessel or intracompartmentally. Each of these sites may have unique characteristics. For example, subcutaneous deposition results in lower mortality (Russell, 1983) but may cause more local toxicity. This variation in absorption is substantiated by clinical data indicating that the onset of effects after crotalid snake bite can be delayed by several hours (Hurlbut *et al.*, 1988).

It is often asserted that venom is absorbed via the lymphatic circulation. Some research in animals supports this conclusion, but clinical observation suggests that there must also be other routes of absorption. For example, it is not uncommon for systemic clinical effects to develop within minutes, a period too rapid for the effects to be due to lymphatic absorption alone. Thus, an antivenom must penetrate to sites of venom distribution as well as deposition.

Despite the marked heterogeneity of snake venom, each venom seems to contain only a few fractions that are responsible for its toxic effects. Some venoms are dominated by one or a few components, while others contain numerous toxic fractions. Thus, there may be a limited number of clinically important components that require neutralization. Nevertheless, this complexity is much greater than that encountered in typical chemical drugs, which generally affect one pathophysiological process.

Requirements of the ideal antivenom

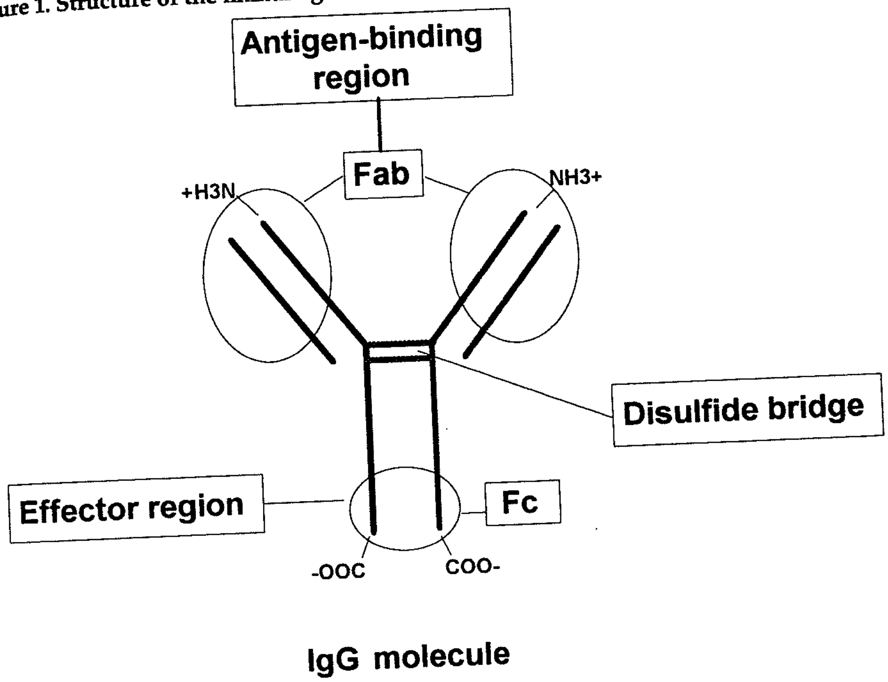
The essential qualities of any drug are efficacy and safety; other important qualities are acceptable cost and ease of administration. A clear understanding of the process of snake venom poisoning can lead to a precise definition of antivenoms that have these qualities. The efficacy of an antivenom is influenced by several factors. First, it is crucial to understand that binding of a venom component does not necessarily mean neutralization (Lamb, 1902). In the case of pit vipers, the antivenom should neutralize the venom components that cause pain, swelling, fibrinogenolysis and thrombocytopenia. Ideally, the antivenom should penetrate to the site of venom deposition and neutralize its components before they produce local injury or are absorbed into the systemic circulation. One physicochemical factor that promotes tissue penetration is small relative molecular mass.

Once effective neutralizing antibodies are produced, the safety of the product becomes important. In the early part of this century, hyperimmune animal sera were administered without processing. While these products were often effective, they produced an unacceptable rate of acute and delayed complications. Subsequently, animal sera were developed that were partially purified to remove unwanted, unnecessary components that do not neutralize venom components; however, these are still rather primitive medications and retain many of the drawbacks of their predecessors (Landon *et al.*, 1995).

Antivenin (Crotalidae) Polyvalent [Wyeth] is a good example of the evolution of antivenom products. The active component of the Wyeth product and many other antivenoms and antitoxins is immunoglobulin (Ig) G. IgG is a protein molecule that binds an antigen at one end and binds to mast cells or vascular endothelium at the effector end (Figure 1). Antivenin (Crotalidae) Polyvalent was an advanced product when it was introduced in 1956 because ammonium sulfate precipitation was used to eliminate some of the unwanted serum components. Today, it is still produced by the original method of equine hyperimmunization with a mixture of four crotalid venoms. The hyperimmune serum is harvested periodically and subjected to ammonium sulfate precipitation (Wyeth Laboratories, 1961). The product is then lyophilized and packaged. Although Wyeth claimed that their process '... eliminates most of the animal protein, and lessens liability to untoward reactions....', the final Wyeth product has been shown to contain 15.4–25.6% IgG and also IgM, albumin and other components of horse serum (Sullivan, 1987)

These contaminants are excellent inducers of allergic reactions. Antivenom-induced allergic reactions fall into two categories: anaphylaxis, a type-I hypersensitivity reaction, and serum sickness, a type-III reaction. All heterologous sera—indeed nearly all medications of any type—are capable of causing anaphylaxis, a cascade of events that may produce effects including bronchoconstriction and hypotension. Anaphylaxis may be overwhelming and cause death despite appropriate therapy. It has been recorded many times after

Figure 1. Structure of the immunoglobulin G (IgG) molecule



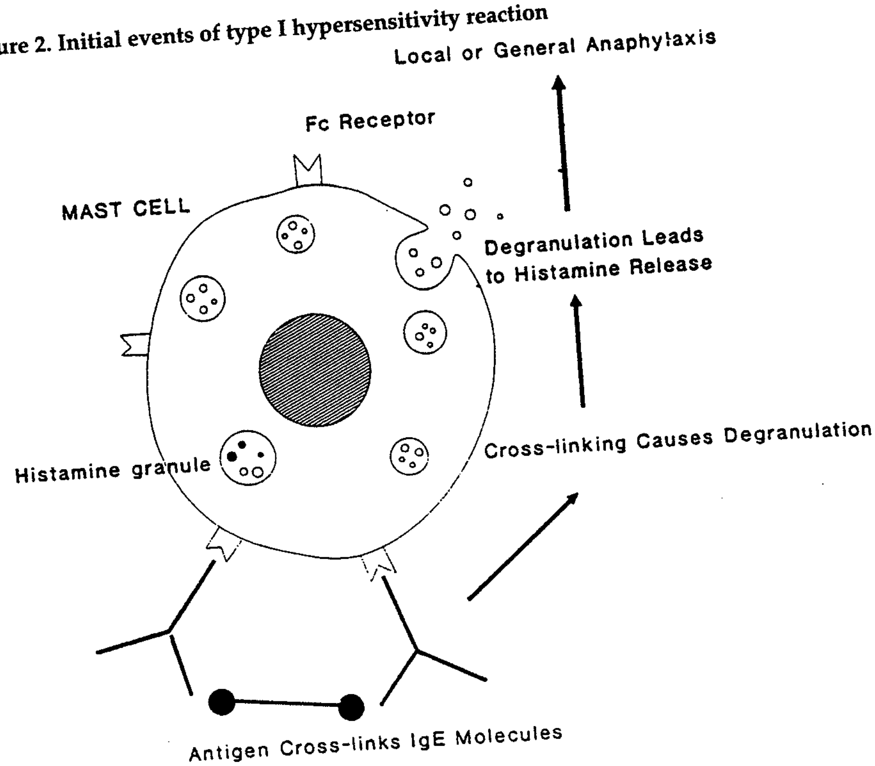
IgG is a protein composed of two heavy chain and two light chains. The molecule is divided into an antigen-binding domain (Fab) and an effector region (Fc). The heavy chain spans both regions; the light chain is involved only in the Fab.

the administration of antivenoms, although the exact incidence has not been well documented. It probably occurs in less than 15% of cases (Jurkovich *et al.*, 1988) but constitutes the major fear of physicians with regard to antivenoms.

Several components of sera may induce anaphylaxis. In a sensitized individual, heterologous proteins are recognized and bound by immunoglobulins. The recipient's IgE molecules, bound to the surface of mast cells (Figure 2), play a central role in the process. Binding of an antigen by IgE leads to cross-linking and activation of the mast cell. This causes mast cell degranulation, thereby initiating a cascade of events which culminates in an immediate inflammatory response. The allergic response may be localized or systemic. In essence, the Fc portion of IgE acts to transmit information from the Fab portion of IgE ('A foreign molecule has been captured.') to the mast cell ('It is time to release mediators.').

A related process is the anaphylactoid reaction. This is a process by which mast cells are directly stimulated to release mediators, presumably by non-neutralizing proteins such as IgG aggregates or preformed, complement-like components of immune sera (Sutherland, 1977).

Figure 2. Initial events of type I hypersensitivity reaction



From Landon *et al.* (1995)
Binding of a foreign antigen produces cross-linking of immunoglobulin E molecules on the surface of mast cells. This produces degranulation and initiates a sequence of events that may produce anaphylaxis.

Renal injury is another concern. A bolus infusion of 2.0 mg/kg body weight (bw) digoxin-specific ovine antigen-binding domain (Fab) caused a transient decrease in creatinine clearance in a rat model (Timsina & Hewick, 1992). A much larger dose of Fab (3.2–5.3 g/kg bw) decreased creatinine clearance in dogs, but only for a few days (Keyler *et al.*, 1991). Similar effects have not been reported in humans. For example, the administration of a 10-vial dose of Antivenin (Crotalidae) Polyvalent would deliver 15–20 g of protein intravenously (Sullivan & Russell, 1983). Since 10 vials is a typical starting dose, occasionally followed by up to 40 vials or more, renal injury would seem to be a concern. The effect on renal function has never been subjected to detailed study; nevertheless, there is no case report in the medical literature of renal failure induced by crotalid antivenom. The Wyeth product is a mixture of proteins, however, none of which is Fab. Is there something unique with regard to Fab and renal injury?

The data available on Fab administration to humans are not extensive in comparison with those on Antivenin (Crotalidae) Polyvalent, but they are

reassuring. Renal toxicity has not emerged as a problem after the administration of digoxin immune Fab (Antman *et al.*, 1990; Kirkpatrick, 1991). The total Fab dose obtained from the administration of 20 vials of Digibind would amount to only 11.4 mg / kg bw for a 70-kg person. In a North American trial of a new affinity-purified Fab for crotalid snake venom poisoning, a dose of 40–80 mg / kg bw could be reached without adverse renal effects. It does not appear likely, therefore, that therapeutic doses of Fab will be toxic to humans, particularly if they have normal renal function. There may be other therapeutic applications of Fab, however, that will require larger doses (Sullivan, 1986), and potential renal toxicity should be kept in mind.

Other desirable traits of an antivenom are reasonable cost and ease of administration. It is difficult to estimate an appropriate cost for an antivenom. One approach is to compare the cost to that of currently available antivenoms. Antivenin (Crotalidae) Polyvalent costs US$ 150–200 per vial in the United States. The cost for full treatment of a moderate-to-severe case of crotalid snake venom poisoning, therefore, would reach US$ 2000 and could exceed US$ 6000 or more, depending on the severity of envenoming. Another approach would be to compare the cost of a new antivenom with the cost of treating a snake bite without antivenom. Depending on the species involved, the cost for a typical snake bite (excluding dry bites) in the United States would range from a few hundred dollars for a minimal bite treated without antivenom to tens of thousand of dollars for a severe bite, which would require several days in an intensive care unit. Obviously, the relative costs would vary widely by country, making it difficult for industrialized nations to produce antivenom for countries with low health care costs.

Ease of administration is easier to define. Since delay to antivenom administration decreases the effectiveness of the antivenom (Dart *et al.*, 1988), an antivenom should be administered as early as possible after a bite. Thus, the optimal time for antivenom administration may be before arrival at a hospital, especially when transport time is prolonged. The product would therefore have to be resistant to decomposition caused by shaking and temperature variation. Furthermore, new products should be soluble within a few minutes, in contrast to the 30–45 min currently required for solubilizing traditional antivenoms.

Fab as antivenoms

Some antivenoms are simply immune sera harvested from horses, sheep or goats. Others are partially purified products of immune sera (Wyeth Laboratories, 1961). As noted above, partially purified hyperimmune animal sera are often effective but lack the safety profile desired in a therapeutic product. Since the only known life-threatening reaction to antivenom is anaphylaxis, efforts have focused on eliminating this problem. One approach has been to reduce components other than the desired venom-specific IgG. For example, Sullivan and Russell (1982) demonstrated that affinity purification can be used to isolate the venom-specific

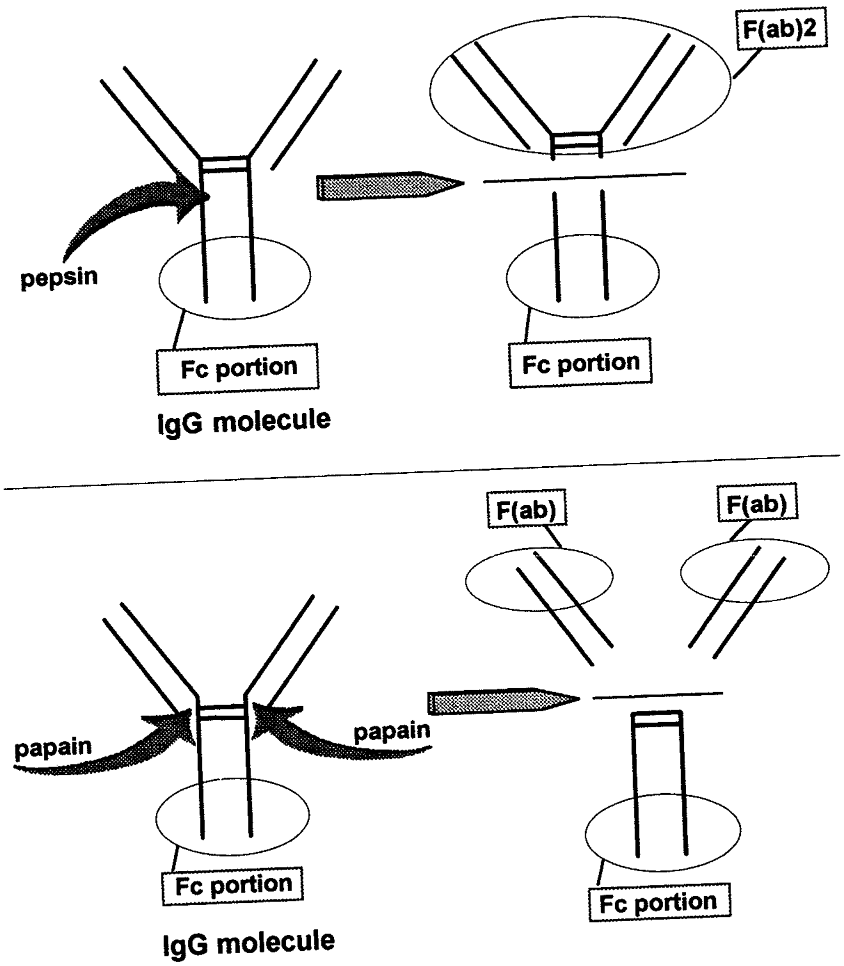
IgG molecules in hyperimmune horse serum. The resulting antivenom was more potent than the Wyeth product from which it was derived and had much better performance in the assays for anaphylaxis in guinea-pigs and histamine release from leukocytes in humans (Russell *et al.*, 1985). Such purification alone, however, leaves the equine IgG(T) as the active component of the antivenom. As equine serum contains a structural variant of IgG (Weir & Porter, 1966) that is particularly immunogenic (Cartledge *et al.*, 1992), many antivenoms have been produced in sheep or goats.

There is another disadvantage to the use of purification alone to produce a safer antivenom. Affinity purification may solve the safety issue, but it does little to address other concerns. The relative molecular mass of IgG is about 150. Thus, its distribution is mainly limited to the circulating blood volume, particularly immediately after administration. An antibody with a smaller molecule might allow more rapid distribution from the blood to the site of venom deposition. In addition, even pure heterologous IgG is probably capable of inducing anaphylaxis. Fortunately, the enzyme papain cleaves IgG into three parts: one fragment termed Fc and two fragments termed Fab (Figure 3). Fab has several desirable characteristics. First, despite the disruption of the IgG molecule, Fab retains the ability to bind and, more importantly, neutralize the effects of a venom component. Second, its relative molecular mass is only 50, resulting in a markedly increased volume of distribution. In theory, therefore, Fab are capable of better tissue penetration. In addition, the lower relative molecular mass means that the unbound Fab can be excreted through the kidneys. Finally, the Fc portion is removed from the final product, thus reducing the incidence of allergic reactions.

Another approach that has been used for several recently developed antivenoms is the use of $F(ab)_2$ preparations. Digestion of IgG with pepsin cleaves IgG to produce one Fc and one $F(ab)_2$. The $F(ab)_2$ is divalent, containing two binding sites for antigen (Figure 3). This technique shares with papain digestion the advantage of allowing subsequent removal of the Fc portion. The $F(ab)_2$ has a relative molecular mass of 100, so that its tissue penetration may be poorer than that of Fab. Despite the theoretical advantages over IgG antivenoms, $F(ab)_2$ products have not proven as safe as anticipated: While some are safer than IgG products, investigators report reaction rates of 30–84% for others (Malasit *et al.*, 1986; Cardoso *et al.*, 1993).

Regardless of this promising theory and of the results in experimental animals, the safety and efficacy of Fab preparations in humans must be documented before they can be widely used. The best evidence of their safety comes from work not with an antivenom but with digoxin immune Fab (Digibind®). Digoxin Fab is produced by the same general method as an antivenom product: A group of animals is immunized against a digoxin–adjuvant complex, and their serum is harvested; then, the anti-digoxin Fab is isolated, lyophilized, packaged and administered to humans. The production of digoxin immune Fab involves three techniques to produce safe antibodies: it is produced in sheep instead of horses, it is treated with papain to produce Fab from IgG, and it undergoes affinity

Figure 3. Production of Fab and $F(ab)_2$ fragments from immunoglobulin G (IgG)



Digestion with the enzyme pepsin cleaves the IgG molecule proximal to a disulfide bond that attaches the $F(ab)_2$ fragment from the Fc fragment. The result is one Fc and one $F(ab)_2$. Digestion with papain cleaves the IgG molecule distal to the disulfide bond and thereby separates the two Fab fragments. The result is one Fc and two Fab.

purification to eliminate unwanted portions of the sheep serum. True anaphylaxis has not been observed in almost 1000 patients treated in trials or in a post-marketing surveillance study (Antman *et al.*, 1990; Kirkpatrick, 1991).

There is now evidence in humans bitten by snakes as well. An ovine Fab antivenom for the treatment of *Vipera berus* envenoming has been shown to be safe and effective. Patients treated with the antivenom developed less extensive oedema and anaemia than patients treated in the traditional manner, and the

duration of hospitalization was shorter than for control patients. Only three allergic reactions were seen: one patient developed an acute reaction consisting of simple urticaria, and two patients developed serum sickness (Karlson-Stiber & Persson, 1995). Furthermore, a prospective multicentre clinical trial is now in progress in the United States to demonstrate the safety and efficacy of an ovine, affinity-purified Fab for the treatment of poisoning by North American crotalid snakes. The study is supported by an Orphan Product Development grant from the US Food and Drug Administration and by Therapeutic Antibodies, Inc. (Nashville, TN). The antivenom is prepared by immunizing sheep to one of four venoms: eastern diamondback (*C. adamanteus*), western diamondback (*C. atrox*), Mojave rattlesnake (*C. scutulatus scutulatus*) and cottonmouth snake (*Agkistrodon piscivorus*). The result is four separate monospecific antivenoms, which are then pooled to create one polyspecific antivenom for use throughout the United States. The antivenom was remarkably more potent than Antivenin (Crotalidae) Polyvalent in trials in animals (unpublished data). It has been administered to 10 human subjects with minimal to moderate envenoming. In order to qualify for enrolment, each subject had to develop evidence of progression of venom effect. After enrolment, each patient received four vials of the ovine antivenom; the dose could be repeated once at the discretion of the investigator. All subjects had follow-up visits at 7 and 14 days. All 10 patients showed an effective response to the antivenom (Dart *et al.*, 1994). Most importantly, none of the subjects developed an acute allergic reaction or serum sickness.

Future concerns and possibilities

One of the most frustrating aspects of venomous snake bite is the inability to administer antivenom in the field. A frequent question from both individuals and organizations planning expeditions into remote areas is, 'What should I take on this expedition that will allow us to give antivenom in the field?' In the United States, antivenom cannot be administered safely outside of a health care facility, as anaphylaxis can be so devastating that equine antivenom should be administered only in a fully equipped intensive care facility. If an effective antivenom were safe enough for administration in the field, however, treatment could be initiated immediately and our concerns about first aid measures could become a quandary of the past. While they may take many years to develop, Fab antivenoms might be made safe enough to be administered intravenously or intramuscularly in the field.

Although much less expensive than their 'high-tech' cousins, the monoclonal antibodies, polyclonal antibodies are more expensive than many traditional drugs. While the cost–benefit analysis of medical care with and without antivenoms is favourable in countries where health care costs are high, the opposite may be true in developing countries. Thus, one challenge must be to find methods to produce polyclonal Fab in a more cost-effective manner.

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Cross reactivity of three antivenoms against North American snake venoms

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Abstract

The antivenom in the United States today is in short supply, expensive and may not even be the most effective in neutralizing venoms from snakes in certain geographical locations. The ED_{50} is considered to be the best indicator of antivenom efficacy, however, other tests are needed. In this study, three antivenoms (Antivipmyn (Fab_2H), Crotalidae Polyvalent Immune Fab (Ovine) (FabO) and UCV (FabV) were used to test the effectiveness of neutralization of eight venoms (*Agkistrodon piscivorus piscivorus, Bothrops asper, Crotalus adamanteus, C. durissus durissus, C. horridus atricaudatus, C. h. horridus, C. atrox*, and *C. molossus molossus*). Four different assays were used to study the efficacy of the antivenoms: the antihemorrhagic, antigelatinase, antifibrinolytic and antihide powder azure. Fab_2H antivenom was more effective in neutralizing the enzymatic activities of these eight venoms than FabO and FabV antivenoms.

Keywords: Antivenom; Venom; *Crotalus*; *Bothrops*; Antihemorrhagic; Hemorrhagic; Fibrinolytic; Gelatinase; Hide powder

1. Introduction

Mortality from snake envenomation has not been a major problem in the United States, since the death rate in the United States is approximately eight per year. However, loss of limbs, permanent disabilities and slow recovery time due to envenomation are serious problems. The best and most acceptable form of treatment is with the use of antivenom, however, the antivenom must be administered as soon as possible since tissue damage cannot be reversed.

There are 44 different subspecies of venomous snakes in the United States. A majority of the venomous snakes are pit vipers that have complex mixtures of toxins and are difficult to neutralize. Geographical variation of snake venom is well documented in the literature (Adame et al., 1990; Glenn and Straight, 1977, 1978, 1989; Johnson et al., 1987). Because of the geographical variation, physicians encounter different clinical situations with each snakebite victim. Snakes use their venom for capturing their prey and therefore, fast acting venom would enhance a snake's ability to immobilize the prey quickly. This immediate response creates medical emergencies when humans are envenomated. In most cases, the envenomating species is seldom known and there is a delay before antivenom can be delivered. Ideal antivenom would be one readily available to a physician that can be administered immediately, and cross reacts with a large number of venoms and minimizes allergic reactions in humans.

There are many antivenoms produced throughout the world (Theakston and Warrell, 1991) and each is produced in a different way and for a different purpose. The different laboratories producing antivenoms have very different procedures such as (1) the type of animals used, (2) snake venoms used, (3) immunization protocols, (4) cleavage of the IgG molecule, and (5) purification procedures. Inspite of these differences, most antivenoms

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will cross-react with venoms that are not used in the immunization protocol. There are many important considerations in producing a polyvalent antivenom. However, the most important is to start with the correct combination of venoms that will best represent the area where the antivenom will be used, and this will require the development of different assays.

The effective dose of antivenom that will protect 50% of a mouse population (ED_{50}) is considered the most effective way of analyzing the efficacy of antivenom. However, other tests are needed to analyze the overall efficacy of antivenom. The focus of this study was to measure neutralization of hemorrhagic, fibrinolytic, gelatinase and hide powder azure activities in eight snake venoms with three different antivenoms using assays other than the ED_{50}.

2. Methods

2.1. Venoms

Eight venoms (*Agkistrodon piscivorus piscivorus, Bothrops asper, Crotalus adamanteus, C. durissus durissus, C. horridus atricaudatus, C. h. horridus, C. atrox*, and *C. molossus molossus*) were used to study the efficacy of neutralization with three antivenoms. Venoms from *C. h. horridus, C. h. atricaudatus* and *C. m. molossus* were obtained from the Natural Toxins Research Center (NTRC) at Texas A&M University-Kingsville, TX. *C. h. horridus* was an equal mixture of venoms #010-327-277, 010-875-780 and 010-595-578, the venom of *C. h. atricaudatus* was an equal mixture of venoms #011-522-004 and 010-782-102, and *C. m. molossus* venom was an equal mixture of venoms #011-064-286, 010-526-346, 011-087-008, and 010-861-812. By coding the venoms with pit tag numbers the same venoms can be used in future studies and location data, age of the snake, sex and venom characteristic can be traced with pit tag numbers. This information can be obtained from the NTRC database (http://ntri.tamuk.edu/serp/index.html) by the nine digit number assigned to the snakes (Perez et al., 2001).

C. atrox venom was a pooled sample obtained in Big Springs, TX. Pooled venom from *A. p. piscivorus* was obtained from Biotoxins, St Cloud, FL, pooled venom from *C. adamanteus* was obtained from SIGMA and venoms from *C. d. durissus* and *B. asper* were obtained from Bioclon, Inc., Mexico. The venom samples were centrifuged at $500 \times g$ for 10 min and filtered using a Millipore Millix HV 0.45 μm filter unit. All venoms were lyophilized and stored at −90 °C. The NTRC has over 360 snakes, when possible, the snakes were chosen for their venom to represent both genders, age and size differences.

2.2. Antivenoms

Antivipmyn (Fab_2H) is an equine origin antivenom produced by Instituto Bioclon in Mexico (lot no. B-2E-05, exp. date: May, 2006). The snake venoms used to produce the Fab_2H are *Crotalus durissus* and *B. asper* and is $(Fab')_2$ fragment. The second antivenom used was Crotalidae Polyvalent Immune Fab (Ovine) (FabO) that has an ovine origin and produced by Therapeutic Antibodies, Inc., London, England (lot no. CRO/140/008/001, exp. date: not available). The snake venoms used to produce FabO were *Crotalus atrox* (Western Diamondback Rattlesnake), *C. adamanteus* (Eastern Diamondback Rattlesnake), *Crotalus scutulatus scutulatus*(Mojave Rattlesnake), and *A. p. piscivorus* (Eastern Cottonmouth). FabO is an affinity purified Fab fragment. The third antivenom was UCV (FabV) that is an equine origin produced at the Universidad Central de Venezuela in Caracas, Venezuela by the Department of Pharmacy (lot no. 02187, exp. date: Sept. 1989). The snake venoms used to produce FabV are *Bothrops colombiensis, B. venezuelensis, B. atrox, Crotalus durissus cumanensis* and *C. vegrandis*. The serum contains Fab fragments cleaved with papain and purified with DEAE-Cellulose.

2.3. Hemorrhagic assay

The minimal hemorrhagic dose (MHD) for crude venom was determined by using a modification of the Omori-Satoh et al. (1972) method. Crude venoms were diluted two-fold into various concentrations of which 0.1 ml of each dilution was injected intracutaneously into the back of a depilated New Zealand white rabbit (*Oryctolagus cuniculus*) to test for hemorrhagic activity. After 24 h, the rabbit was sacrificed and the hemorrhagic spots were measured. An MHD is the amount of venom protein (μg) that produces a 10 mm hemorrhagic spot. This assay was repeated three times.

2.4. Antihemorrhagic assay

Equal amounts of two MHD of crude venoms were mixed with the antivenoms and incubated for 1 h at 25 °C. The back of a New Zealand rabbit was depilated and 0.1 ml of each dilution was injected intracutaneously. The rabbit was sacrificed after 24 h and the hemorrhagic spots were measured if present. The antihemorrhagic dose (AHD) is defined as the amount (μg) of antivenom inhibiting 50% of 1 MHD of the various snake venoms. The lower the number the more efficient the antivenom. This assay was repeated three times.

2.5. Fibrinolytic assay

A method modified from Bajwa et al. (1980) was used to measure fibrinolytic activity of the fractions

obtained from the venoms. Fibrinogen solution (9.4 mg/ml, 300 μl) and thrombin solution (38.5 U/ml, 10 μl) were added to each well of a 24-well plate. After each well contained both solutions, the plate was shaken gently. The solution in the plate was allowed to solidify at room temperature. The plate was then incubated for 3 h at 37 °C. Twenty microliters of various concentrations of crude venom were added individually in the center of their corresponding wells and incubated overnight at 37 °C. Seven hundred microliters of 10% trichloroacetic acid (TCA) were placed in each well, then decanted after 10 min. Positive results were obtained by a clearing of the fibrin. The minimal fibrinolytic dose (MFD) was determined as the amount of venom protein (μg) that will clear a 5 mm area in the fibrin clot. This assay was repeated three times.

2.6. *Antifibrinolytic assay*

Equal amounts of two MFD of crude venom were incubated with various two-fold serial dilutions of the three antivenoms. The antifibrinolytic dose is defined as the amount (μg) of antivenom inhibiting the degradation of fibrin by one MFD. The lower the number the more efficient the antivenom. This assay was repeated three times.

2.7. *Gelatinase assay*

A method modified from Huang and Perez (1980) was used to test gelatinase activity of crude venom. Twenty microliters of various concentrations of crude venom were placed on a Kodak X-OMAT scientific imaging film with gelatin coating. Hydrolysis of gelatin on the X-ray film was determined by washing the film with tap water after 4 h incubation at 37 °C in a moist incubator. A positive result was noted if there was clearing of the gelatin on the X-ray film. The minimal amount of venom that causes a clear zone on a Kodak X-OMAT scientific imaging film is defined as the minimal gelatinase dose (MGD). This assay was repeated three times.

2.8. *Antigelatinase assay*

Equal amounts of two MGD were then incubated with various two-fold serial dilutions of the three antivenoms. The antigelatinase dose (AGD) is defined as the amount (μg) of antivenom inhibiting the clearance of the gelatin on the X-ray film by one MGD of each of the venoms. The lower the number, the more efficient the antivenom. This assay was repeated three times.

2.9. *Hide powder azure assay*

A modified method of Rinderknecht et al. (1968) was used to test proteolytic activity. Eight milligrams of hide powder azure were mixed with 2 ml of 0.02 M Tris–HCl buffer, pH 8.0, and 100 μl of various concentrations of crude venom were added to the vial. Each vial was incubated for 1 h at 37 °C and agitated at 5 min intervals. After incubation, each vial was centrifuged at 420 *g* for 5 min. The supernatant was transferred into a different vial and the absorbance measured at 595 nm. The hide powder azure was ground with a mortar and pestle to obtain uniform particles. An absorbance reading higher than 0.1 gave positive results. The minimal amount of venom that causes an absorbance reading of 0.1 at 595 nm is defined as the minimal hide powder dose (MHPD). This assay was repeated three times.

2.10. *Antihide powder azure assay*

The minimal amount of venom that causes an absorbance reading of 0.1 at 595 nm was determined for each of the eight venoms using hide powder azure. Equal amounts of two MHPD were then incubated with various two-fold serial dilutions of the three antivenoms. The antihide powder azure dose (AHPD) is defined as the amount (μg) of antivenom inhibiting an absorbance reading of 0.1 of one MHPD of each of the venoms. The lower the number the more efficient the antivenom. This assay was repeated three times.

3. Results

All venoms contained hemorrhagic activity (Table 1). Fab_2H antivenom was capable of neutralizing the hemorrhagic activity of all the venoms while FabO and FabV only neutralized 50% of the venoms.

Fibrinolytic activity was present in 62.5% of the venoms (Table 2). None of the antivenoms were able to neutralize the fibrinolytic activity of *A. p. piscivorus* venom. Fab_2H was able to neutralize the fibrinolytic activity of four venoms, while FabV antivenom neutralized the fibrinolytic activity of three venoms and FabO neutralized the fibrinolytic activity of only two venoms.

Gelatinase activity was present in all the venoms (Table 3). Fab_2H was able to neutralize the gelatinase activity of all the venoms. Neither FabO nor FabV were able to neutralize the gelatinase activity of *A. p. piscivorus*, *C. d. durissus* and *C. m. molossus* venoms.

Hide powder azure activity was present in seven out of eight venoms used in this study (Table 4). *C. h. horridus* venom was the only venom void of hide powder azure activity. Neither FabO nor FabV were able to neutralize the hide powder azure activity of the venoms used in this study. Fab_2H was able to neutralize the hide powder activity in only three of the venoms (*B. asper*, *C. adamanteus* and *C. m. molossus*).

Table 1
MHD for eight snake venoms and the AHD of three antivenoms

Venom	MHD (μg)	Fab_2H AHD (μg)	FabO AHD (μg)	FabV AHD (μg)
A. p. piscivorus	26 ± 1.4	**53 ± 0**	PN	–
B. asper	5.6 ± 0.56	**26.5 ± 12.3**	–	–
C. adamanteus	0.3 ± 0.18	**1 ± 0.2**	4.4 ± 0	53 ± 0
C. d. durissus	50 ± 7.1	**425 ± 0**	–	–
C. atrox	2.5 ± 0	26.5 ± 0	**6.6 ± 3/1**	212 ± 0
C. h. atricaudatus	37.5 ± 0.71	**212 ± 0**	–	–
C. h. horridus	5.6 ± 0.28	**4.4 ± 0**	6.6 ± 0	106.5 ± 50.2
C. m. molossus	12.5 ± 0	**35 ± 0**	283 ± 0	283 ± 0

MHD: the amount of venom protein (μg) injected into the back of depilated rabbit causing a 10 mm hemorrhagic spot in diameter. Antivenoms were at a starting concentration of 8.5 mg/ml. AHD: antihemorrhagic dose—the amount of antivenom (μg) that neutralizes 50% of 1 MHD of venom. The AHD is calculated by dividing the starting concentration of antivenom by the dilution that neutralizes 50% of 1 MHD and then multiplying by the amount of volume injected into the back of a depilated rabbit. PN: partial neutralization of venom. The appearance of the hemorrhagic spot appeared to be scattered and not a reduction of the hemorrhagic area. –: indicates that the MHD was not neutralized with equal volume of antivenom at a concentration of 8.5 mg/ml. Values in bold represent the antivenom most effective in neutralizing activity. The MHD and AHD for each venom and antivenom were repeated three times.

Table 2
MFD for eight snake venoms and the AFD of three antivenoms

Venom	MFD (μg)	Fab_2H AFD (μg)	FabO AFD (μg)	FabV AFD (μg)
A. p. piscivorus	11.2 ± 0	–	–	–
B. asper	1.25 ± 0	**5.3 ± 0**	21.2 ± 0	42.5 ± 0
C. adamanteus	na	na	na	na
C. d. durissus	4.5 ± 1.5	**10.6 ± 0**	–	42.5 ± 0
C. atrox	5.7 ± 0	**10.6 ± 0**	21.2 ± 0	85 ± 0
C. h. atricaudatus	na	na	na	na
C. h. horridus	na	na	na	na
C. m. molossus	4.7 ± 1.6	**42.5 ± 0**	–	–

MFD: the amount of venom protein (μg) that causes a 5 mm clearing zone on a fibrin plate. Antivenoms were at a starting concentration of 8.5 mg/ml. AFD: antifibrinolytic dose-the amount of antivenom (μg) that neutralizes 1 MFD of venom. The AFD is calculated by dividing the starting concentration of antivenom by the dilution that neutralizes 1 MFD and then multiplying by the amount of volume added to the fibrin plate. na: not applicable due to lack of venom activity. –: indicates that the MFD was not neutralized with equal volume of antivenom at a concentration of 8.5 mg/ml. Values in bold represent the antivenom most effective in neutralizing activity. The MFD and AFD for each venom and antivenom were repeated three times.

Table 3
MGD for eight snake venoms and the AGD of three antivenoms

Venom	MGD (μg)	Fab_2H AGD (μg)	FabO AGD (μg)	FabV AGD (μg)
A. p. piscivorus	4.6 ± 1.6	**42.5 ± 0**	–	–
B. asper	2.5 ± 0	**10.6 ± 0**	42.5 ± 0	85 ± 0
C. adamanteus	11.6 ± 0	**10.6 ± 0**	42.5 ± 0	5.3 ± 0
C. d. durissus	1.1 ± 0.4	**70 ± 25**	–	–
C. atrox	1.2 ± 0.4	**42.5 ± 0**	85 ± 0	170 ± 0
C. h. atricaudatus	2.8 ± 0	42.5 ± 0	42.5 ± 0	170 ± 0
C. h. horridus	10.7 ± 0	42.5 ± 0	42.5 ± 0	42.5 ± 0
C. m. molossus	1.2 ± 0.4	**85 ± 0**	–	–

MGD: the minimal amount of venom protein (μg) that causes a clearing on the X-ray film. Antivenoms were at a starting concentration of 8.5 mg/ml. AGD: antigelatinase dose-the amount of antivenom (μg) that neutralizes 1 MGD of venom. The AGD is calculated by dividing the starting concentration of antivenom by the dilution that neutralizes 1 MHD and then multiplying by the amount of volume added to the X-ray film. –: indicates that the MGD was not neutralized with equal volume of antivenom at a concentration of 8.5 mg/ml. Values in bold represent the antivenom most effective in neutralizing activity. The MGD and AGD for each venom and antivenom were repeated three times.

Table 4
MHPD for eight snake venoms and the AHPD of three antivenoms

Venom	MHPD (μg)	Fab_2H AHPD (μg)	FabO AHPD (μg)	FabV AHPD (μg)
A. p. piscivorus	112 ± 0	–	–	–
B. asper	83 ± 29	**850 ± 0**	–	–
C. adamanteus	250 ± 0	**425 ± 0**	–	–
C. d. durissus	40 ± 23	–	–	–
C. atrox	42.7 ± 25	–	–	–
C. h. atricaudatus	110.8 ± 0	–	–	–
C. h. horridus	na	na	na	na
C. m. molossus	14.2 ± 0	**850 ± 0**	–	–

MHPD: the minimal amount of venom protein (μg) that causes an absorbance reading of 0.1 or higher at 595 nm. Antivenoms were at a starting concentration of 8.5 mg/ml. AHPD: antihide powder azure dose-the amount of antivenom (μg) that neutralizes 1 MHPD of venom. The AHPD is calculated by dividing the starting concentration of antivenom by the dilution that neutralizes 1 MHPD and then multiplying by the amount of volume added to the hide powder azure. na: not applicable due to lack of venom activity. –: indicates that the MHPD was not neutralized with equal volume of antivenom at a concentration of 8.5 mg/ml. Values in bold represent the antivenom most effective in neutralizing activity. The MHPD and AHPD for each venom and antivenom were repeated three times.

4. Discussion

The focus of this study was to determine cross-reactivity of three antivenoms by using assays other than the ED_{50}. Snake venoms have different HPLC, ET, and enzymatic profiles. Most venoms contain both acidic and basic proteins as determined by ET and ion exchange chromatography (Perez et al., 2001). Three of the snake venoms (*C. m. molossus*, *C. h. atricaudatus* and *C. h. horridus*) in this study were chosen because of the drastic differences in their HPLC, ET, and enzymatic profiles which are list on the NTRCs database (Perez et al., 2001). Venoms from these three species of snakes (*C. m. molossus*, *C. h. atricaudatus* and *C. h. horridus*) were not used in the immunization protocol for the three antivenoms used in this study.

One venom (*C. m. molossus*) is extremely basic containing many proteins with high isoelectric points (pI) greater than 8.0 and highly proteolytic (Sánchez et al., 2001). The hemorrhagic, fibrinolytic, gelatinase and hide powder azure activities of *C. m. molossus* were effectively neutralized by Fab_2H antivenom (Tables 1–4). FabO and FabV neutralized hemorrhagic activity only with very high concentrations of antivenom (Table 1) and were not able to neutralize the other proteolytic activities in *C. m. molossus* venom (Tables 2–4).

Two venoms (*C. h. atricaudatus* and *C. h. horridus*) were extremely acidic having all the proteins with pI less than 6.0 (Perez et al., 2001). The hemorrhagic activity of *C. h. atricaudatus* venom was neutralized by Fab_2H (212 μg) but not neutralized by FabO or FabV antivenoms (Table 1). All three antivenoms neutralized the gelatinase activity of *C. h. atricaudatus* venom (Table 3), however, none of the antivenoms were able to neutralize its antihide powder activity of *C. h. atricaudatus* venom (Table 4). The hemorrhagic and gelatinase activities of *C. h. horridus* venom were neutralized by the three antivenoms, and *C. h. horridus* venom did not contain fibrinolytic nor hide powder azure activities.

All venoms tested were hemorrhagic (Table 1) and five of the eight venoms had fibrinolytic activity (Table 2). Hemorrhagic and fibrinolytic activities play an important role in viperidae snake envenomation, promoting coagulopathies and local and systemic bleeding (Otero et al., 1992; Gutiérrez, 1995). Both the hemorrhagic and fibrinolytic activities need to be neutralized with antivenom. Hemorrhage may be the cause of death of a snake's prey that weighs between 0.020 and 1.0 kg, but few humans bleed to death by envenomation. It does not appear likely that a snake will deliver a dose of venom proportional to the size of an adult human averaging between 70 and 250 kg; therefore, bleeding to death in humans is not an issue. However, immediate neutralization of enzymes causing hemorrhage and tissue damage in humans is an important factor in recovery time, prevention of tissue necrosis, and dispersal of thrombin like enzymes (Anai et al., 2002).

It is interesting that the most effective antivenom in this study, Fab_2H, was made with only two venoms (*C. durissus* and *B. asper*). Both *B. asper* and *C. d. durissus* venoms were good choices to include in the immunization protocol for producing an antivenom, Fab_2H, that neutralizes a wide variety of activities found in venoms from North American snakes. This does not mean that Fab_2H would have the most effective ED_{50} since venoms are complex mixtures of proteins and other toxic factors could cause death.

FabO antivenom was generally less effective in neutralizing hemorrhagic, fibrinolytic, gelatinase and hide powder azure activity in the venoms used in this study (Tables 1–4). Four different venoms were used in the immunization protocols (*C. atrox*, *C. adamanteus*, *C. s. scutulatus*, and *A. p. piscivorus*). *C. s. scutulatus* type-A venom is not considered to be a hemorrhagic venom,

but the other three are hemorrhagic venoms. FabO neutralized hemorrhagic activity in *C. adamanteus* and *C. atrox* venoms, but was unable to completely neutralize hemorrhagic activity in *A. p. piscivorus* venom. FabO was also not able to neutralize the fibrinolytic, gelatinase and hide powder azure activity of *A. p. piscivorus* venom (Tables 2–4). This is surprising since, *A. p. piscivorus* venom was one of the venoms used in the production of FabO. FabO partially neutralized the hemorrhagic activity of *A. p. piscivorus* venom while the two other antivenoms (FabV and Fab_2H) completely neutralized the hemorrhagic activity of this venom (Table 1). In Consroe et al., 1995 study, FabO was able to protect mice from the lethal effects (LD_{50}) of *A. p. piscivorus* venom. A reason for lack of complete neutralization of hemorrhagic activity may be due to geographical differences in snake venom used to produce FabO, or essential antibodies could have been lost during the affinity purification process. Geographical variations in snake venom from snakes of the same species has been reported (Adame et al., 1990; Glenn and Straight, 1977, 1978, 1989; Johnson et al., 1987).

There are advantages in using purified antivenom since non-essential antibodies and proteins could be removed, and less foreign protein would be used in treatment, which should make the antivenom safer. Most purification procedures are never 100% efficient or effective and some of the highest affinity antibody could be lost during purification.

Since antivenom is in short supply and expensive, testing of other antivenoms with assay other than the ED_{50} need to be developed. The assay will help in the selection of proper venoms for immunization protocol and purification procedures. It has been shown that the North American snake venoms cross-reacted with three different antivenoms. Three of these venoms (*C. m. molossus*, *C. h. atricaudatus* and *C. h. horridus*) had significantly different HPLC, ET, and enzymatic activities (Perez et al., 2001). Of these eight venoms, Fab_2H cross-reacted with more North American venoms than the other two antivenoms.

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